FINAL REPORT

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INSTITUTION: Columbia University

GRANT TITLE: Signal Transduction of Inflammation in Atopic Asthma

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OBJECTIVES: The objective of this project is to determine the role of the STAT 6 molecule in allergic inflammatory responses.

APPROACH: The cytokines IL-4 and IL-13 are important for the stimulation of allergic immune responses found in atopic asthmatics. The pleotrophic activities of these cytokines allows them to regulate the function of many cells important in immune responses, such as T and B lymphocytes, mast cells, macrophages, and endothelial cells. The regulation of these cells, by IL-4 and IL-13, involves the stimulation of growth (B and T lymphocytes and mast cells) the regulation of differentiation (e.g. the differentiation of naïve t cells to T helper type 2 cells, B cell Ig class switching to IgE), and the regulation of cellular function (e.g. expression of cell adhesion molecules on endothelial cells). The contribution of each of these activities to the generation of an allergic response remains to be defined. IL-4 and IL-13 both utilize the IL-4 receptor α chain for the activation of intracellular signaling pathways and thereby can activate redundant biologic functions. Two different signaling pathways activated by these cytokines have been well described by others and us. One pathway involves the activation of the IRS-1/2 and PI-3 kinase molecules, which appear to be essential for IL-4 induced cellular proliferation. A second pathway involves the activation of the STAT6 transcription factor. STAT6 is required for several IL-4 induced biologic functions including the differentiation of type 2 helper cells and class switching of B cells to IgE. The goal of this grant was to determine the role of STAT6 in cytokine-mediated signaling events required for allergic immune responses. As described below, we have taken several complimentary approaches to this question.

ACCOMPLISHMENTS (throughout the award period):

1. The Generation of dominant negative mutants of STAT6

Tyrosine phosphorylation of STAT6 in response to IL-4 results in the formation of STAT6 homodimers, which bind specific DNA elements. Although binding sites for STAT6 have been shown to be important for the function of several IL-4 inducible promoters, the role of STAT6 in this activation has not been defined. To determine if STAT6 is a transcriptional activator, different portions of the carboxy terminus of STAT6 were fused to the yeast Gal4 protein DNA binding domain. Analysis of these chimeric Gal4-STAT6 proteins demonstrated that a 140 amino acid proline rich region of the carboxy terminus of STAT6 contains a region that activates transcription. Truncation mutants of STAT6 that lack this domain cannot activate transcription

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13. ABSTRACT *(Maximum 200 words)*

Critical progress has been made in the identification and characterization of cells and mediators involved in allergic inflammation. Accumulating evidence supports the importance of cell adhesion molecule expression as an initiating process in tissue inflammation. Despite progress made to date, much is still unknown about the exact mechanisms responsible for this inflammatory response. Scientists have been working to understand the selective cell recruitment operating in allergic disease with the hope of discovering therapeutic intervention strategies that will prevent the accumulation of unwanted cells in inflamed airways. Research has been directed at developing various approaches to generate specific antagonists. Some approaches under study interupt airway inflammation in its early stages during leukocyte-endothelial interactions. Other approaches inhibit cell recruitment at the endothelial wall. Many studies have been done, both in vivo and in vitro, and the advances that have been made suggest that these therapeutic interventions may be the keys to controlling and, possibly, curing asthma and allergic reactions.

14. SUBJECT TERMS

cell adhesion, allergic rhinitis, atopic allergic conditions, cell biology

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and are capable of repressing transcription stimulated by a wild type STAT6 protein. Strikingly, the ability of IL-4 to induce transcription from the immunoglobulin germline ε promoter is suppressed by over-expression of a carboxy terminal deletion mutant of STAT6. These studies demonstrate that the carboxy terminus of STAT6 contains an activating domain required for the induction of genes by IL

Although the STAT6 truncation mutant can act as a "dominant mutant", we generated an even more potent repressor molecule. This molecule is a fusion protein in which STAT6 is fused with the repressor domain of the KRAB protein. This mutant inhibits IL-4 induced transcription when expressed at low levels. This mutant is more effective at inhibiting the function of endogenous STAT6 then the STAT6 truncation mutant.

2. Generation of mice expressing inducible and tissue specific mutants of STAT6

Our next goal was to generate mice in which we could inhibit the function of STAT6, and thereby IL-4 and IL-13 signaling, in either a tissue specific or an inducible manner. The former mice will allow us to explore how blocking IL-4 signaling in different cells alters the generation of an allergic or asthmatic immune response in vivo. The latter mice will help us determine if blocking IL-4 signaling after an atopic asthmatic response has been established can alter the disease process.

We have examined several different tissue specific promoters for their use in these experiments. We have recently used the ICAM promoter to generate several lines of endothelial cell –specific DN STAT6 mice. We also are injecting oocytes with T cell specific (CD2) and B cell specific ($E\mu V_h$) promoter constructs. The analysis of these mice will occur later this year.

We also attempted to establish mice that express the dominant negative STAT6 construct inducibly. This will allow us to determine the importance of STAT6 activation by IL-4 and IL-13 in an established allergic immune response. This should mimic the situation found in human patients. First, we attempted to establish embryonic stem cell lines that inducibly express the DN STAT6 construct using the tetracycline-inducing system. We thought we could screen through these mice and find clones that had a low basal expression level of the mutant STAT6, and also expresses high levels of the mutant STAT6, when induced. Clones that possess these characteristics would then be injected into blastocysts in order to generate mice. However, after one year of work, we could not get the ES clones to express the construct. Therefore, we are generating two strains of mice to address this question. One strain will express the DN STAT6 construct with the Tet-responsive promoter. The other lines will express the Tet repressor cDNA. When mated together, these mice may allow us to address the importance of STAT6 in a secondary allergic immune response.

3. Determination of the role of the Il-4 receptor α chain in IL-4 signaling

The common γ-chain (γc) is a functional component of the IL-4R, yet cells lacking γc are able to respond to IL-4. This had led to the suggestion that a surrogate γ-chain, which can interact with

the IL-4Rα chain to mediate signaling, is expressed on cells lacking γc. An alternative possibility is that in the absence of γc, the IL-4Rα chain is able to transduce signals by homodimerization. To test this latter possibility, a chimeric receptor containing the extracellular domain of c-kit (the stem cell factor (SCF) receptor) and the cytoplasmic and transmembrane domains of the IL-4Rα chain was generated. Treatment of cells expressing the chimeric receptor kit/IL-4Rα with SCF induces activation of the IL-R4α-associated kinase JAK-1 and the transcription factor STAT6. However, tyrosine, phosphorylation of JAK-3, which associates with γc, is not induced by SCF in these cells. SCF-mediated ligation of kit/Il-4Rα is sufficient to elicit IL-4-specific gene expression, including up-regulation of CD23 and synthesis of germ-line ε transcripts. In the T cell line CTLL2, ligation of kit/IL-4Rα induces cellular proliferation. Finally, in JAK-1-deficient HeLa cells, STAT6 activation by IL-4 is completely abolished. Together, these data demonstrate that the IL-4Rα cytoplasmic domain is sufficient to activate JAK-1 and STAT6 and to induce expression of IL-4 target genes, thus identifying a mechanism by which IL-4 signaling can proceed in the absence of JAK-3 and γc.

Immunoreceptor tyrosine-based inhibitory motifs (ITIMs) have been implicated in the negative modulation of immunoreceptor signaling pathways. The IL-4 receptor α chain (IL-4Rα) contains a conserved sequence motif matching an ITIM consensus within its carboxyl terminal. *In vitro*, a tyrosine-phosphorylated peptide corresponding to the putative human IL-4Rα (hIL-4Rα) ITIM but not its nonphoshporylated counterpart, co-precipitates the SH2-containing tyrosine phosphatase-1 (SHP-1) SHP-2, and SH2-containing inositol 5-phosphatase (SHIP). Upon IL-4 stimulation, SHIP is tyrosine phosphorylated and associates with the IL-4Rα *in vivo*. To investigate the role of the ITIM consensus on IL-4 signaling pathway, 32D myeloid progenitor lines were established stably expressing wild type (WT) hIL-4Rα or mutant hIL-4Rα with the ITIM ablated by deletion (Δ712) or site-directed mutagenesis (Y713F). Significantly, 32D cells expressing mutant hIL-4Rα were hyperproliferative in response to IL-4 when compared to cells expressing WT hIL-4Rα. In contrast, no differences were observed in IL-4 mediated protection from apoptosis. The ability of the ITIM mutants to mediate heightened proliferation correlated with an increased level of IRS-2 and STAT6 tyrosine phosphorylation. These results identify a negative regulatory domain in the IL-4Rα via a functional ITIM, which regulates IRS-2, STAT6 activation and IL-4 induced proliferation.

4. Role of SHIP in IL-4 signaling

The SH2-containing inositol 5-phosphatase (SHIP) has been implicated in the negative modulation of signaling pathways activated by cytokines. However, the contribution of SHIP's catalytic activity in regulating cytokine signaling remains unclear. IL-4 stimulation leads to tyrosine phosphorylation and recruitment of SHIP to the IL-4 receptor, implicating its involvement in IL-4 signaling. In this report, SHIP is shown to associate with additional members of the activated IL-4 signaling complex including IRS-2. SHIP also physically associates with the JAK kinases involved in IL-4 signaling, Jak1 and Jak3. Furthermore, co-expression of SHIP and JAK1 leads to their association and SHIP tyrosine phosphorylation. In functional studies, cells overexpressing wild type SHIP are found to be both hyperproliferative in response to IL-4 and have greater survival when cultured with IL-4, in comparison to parental cells. In contrast, cells expressing a catalytically inactive form of SHIP have the exact opposite

phenotype. These results are the first indication that the catalytic activity of SHIP can act as a positive mediator of growth and survival in cytokine signaling.

CONCLUSIONS:

Our studies have identified several important aspects of Il-4 signaling. First, we have identified the domain of STAT6 required for the activation of transcription by STAT6. STAT6 proteins lacking this domain can act as dominant negative proteins and block IL-4 function. Second, we have determined several important features of the IL-4 receptor α chain. We have shown that homodimerization of the IL-4Rα is sufficient for the activation of IL-4 signaling. We have also identified an inhibitory motif within the IL-4Rα. Finally, we have demonstrated that the SHIP phosphatase is recruited o the IL-4 receptor and appears to play an important role in IL-4 induced cellular proliferation and inhibition of apoptosis.

SIGNIFICANCE:

The experiments described above have provided us with several important and novel findings. First, identification of the transcriptional activation domain of STAT6 will us to explore the mechanism by which this protein activates transcription. This may allow us to identify protein interactions or new proteins important for IL-4 function. These will be potential therapeutic targets for the treatment of atopic asthma. In addition, this has allowed us to generate dominant negative STAT6 proteins that will be important tools for determining the role of STAT6 in the atopic immune response.

Our work with the IL-4 receptor also has several important implications. First, this work demonstrates that the activation of the IL-4Rα chain is sufficient for the activation of STAT6. Again, this suggests that therapeutic agents targeted at disrupting the γC chain of the JAK-3 kinase may not alter IL-4 biology. The identification f the ITIM in the IL-4Rα chain is extremely important as it suggests that there are mechanisms by which cells inhibit their own IL-4 signaling. The identification of the proteins responsible for this function of the IL-4Rα will open new areas of research regarding both the pathogenesis and treatment of atopic asthma. Finally, we have identified SHIP as a positive regulator of IL-4 induced cellular proliferation. Again, this is a novel finding and identifies a new protein that may be involved in the pathogenesis of atopic asthma.

PATENT INFORMATION:

None

AWARD INFORMATION:

1996 Cancer Research Institute Clinical Investigator Award
1996 Appointed as Director, Laboratory of Allergy and Inflammatory Lung Diseases
1997 Member, American Society for Clinical Investigation
1997 Pharmacia Allergy Research Foundation International Award

1997- Appointed as Chief, Division of Pulmonary, Allergy and Critical Care Medicine, Columbia University
1998- Appointed Richard J. Stock Associate Professor of Medicine (Immunology) and Microbiology, Columbia University
1998- Appointed as Director, John Edsall-John Woods Asthma Center
1998 Member, Collegium Internationale Allergologicum

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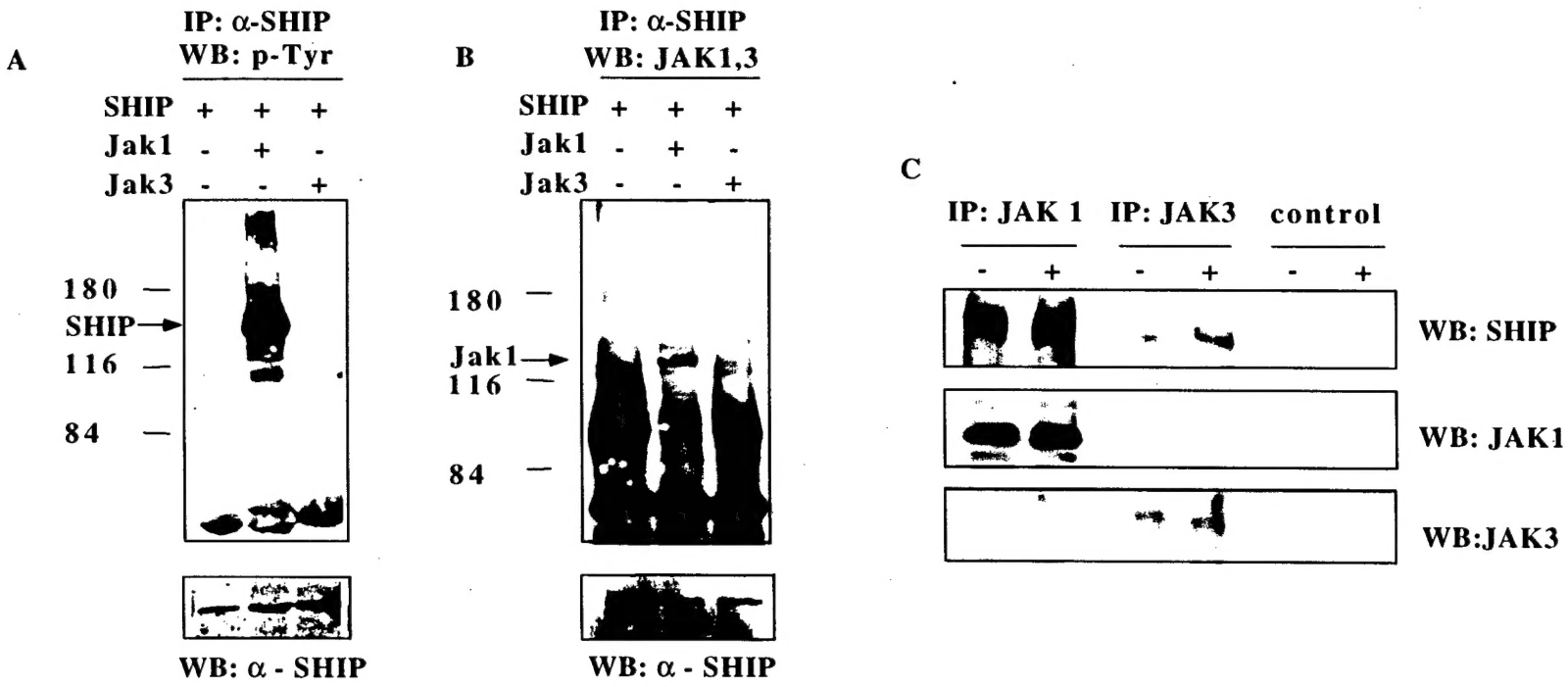
Pan P.-Y. and P. Rothman IL-4 Receptor Mutations. Current Opinions in Immunology (1999) in press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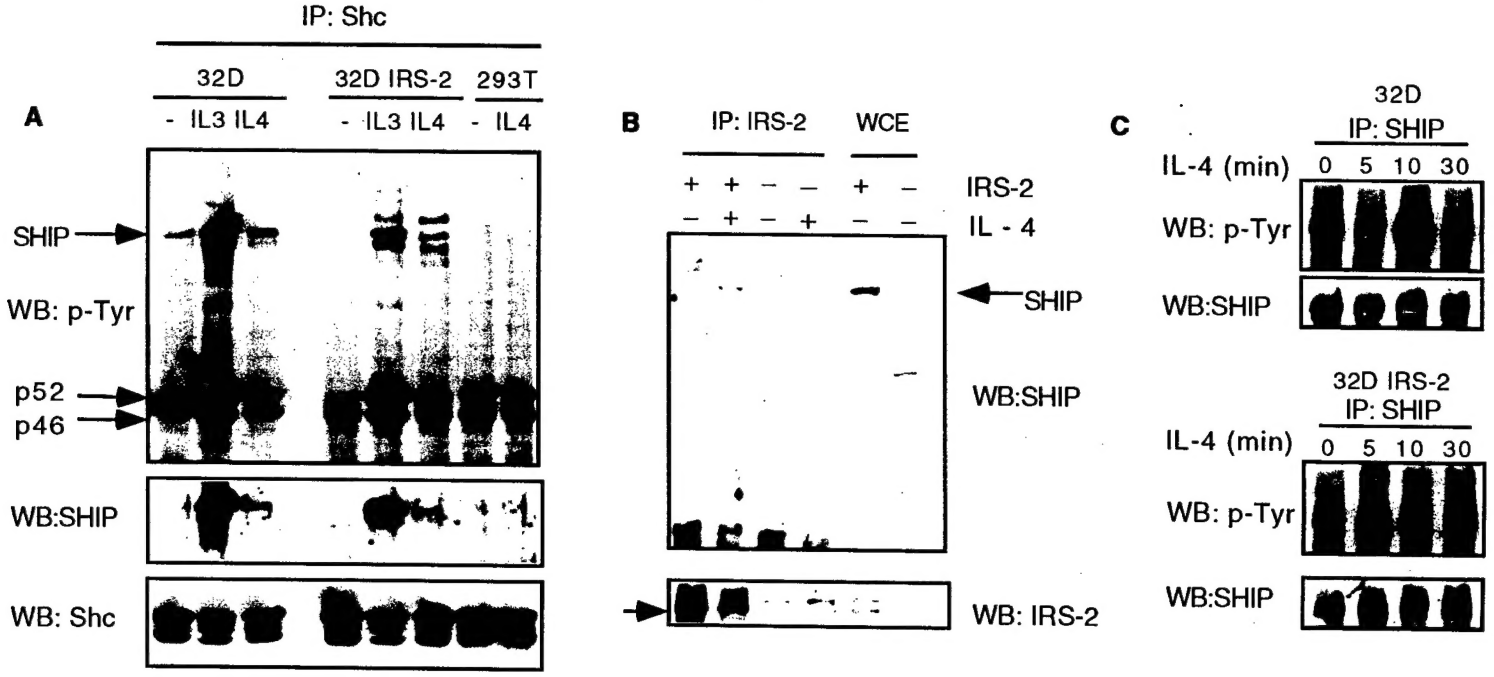
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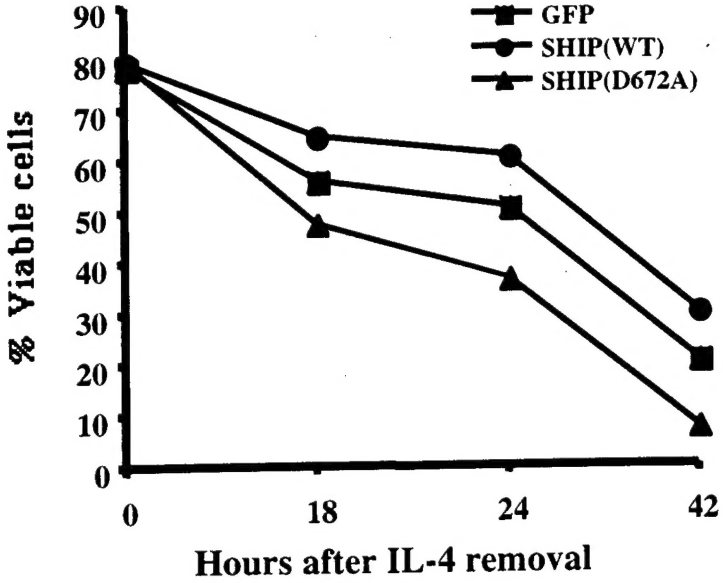
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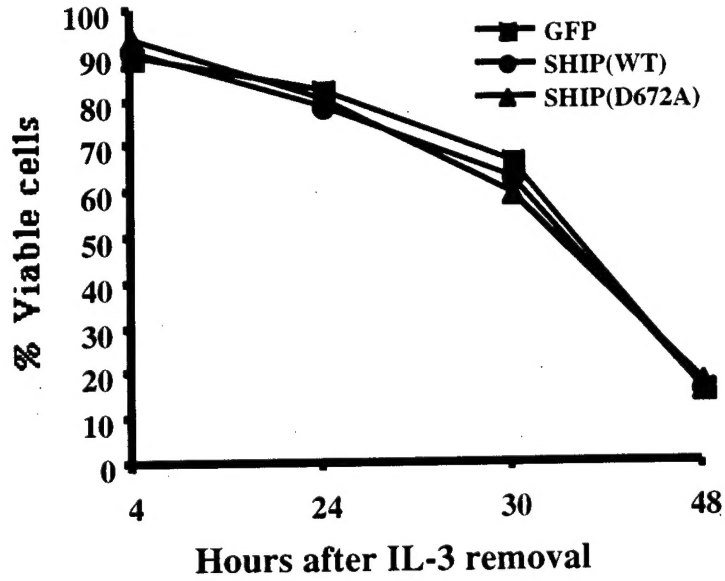
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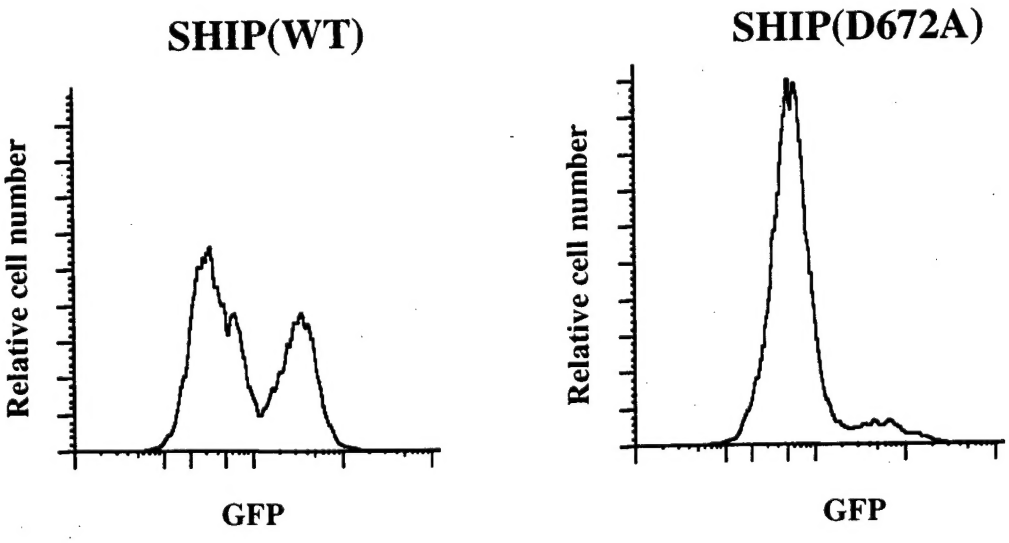
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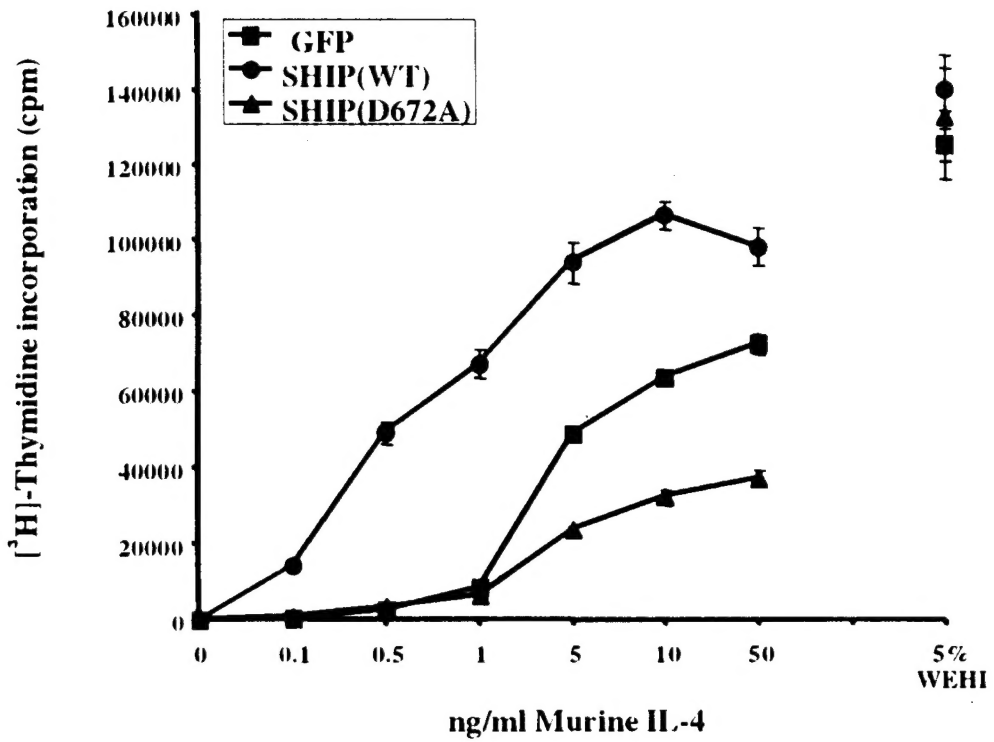
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vector
SHIP(WT)
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control
WB: SHIP
WB: actin

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The SH2-Containing Inositol 5-Phosphatase (SHIP) is a Positive Regulator of IL-4 Mediated Proliferative and Anti-Apoptotic Signaling Pathways

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Running Foot - SHIP is a Positive Regulator of IL-4 Induced Signaling

Key Words – Cytokine, JAK-STAT Signaling

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Summary

The SH2-containing inositol 5-phosphatase (SHIP) has been implicated in the negative modulation of signaling pathways activated by cytokines. However, the contribution of SHIP's catalytic activity in regulating cytokine signaling remains unclear. IL-4 stimulation leads to tyrosine phosphorylation and recruitment of SHIP to the IL-4 receptor, implicating its involvement in IL-4 signaling. In this report, SHIP is shown to associate with additional members of the activated IL-4 signaling complex including IRS-2 and Shc. SHIP also physically associates with the JAK kinases involved in IL-4 signaling, Jak1 and Jak3. Further, co-expression of SHIP and JAK1 leads to their association and SHIP tyrosine phosphorylation. In functional studies, cells overexpressing wild type SHIP are found to be both hyperproliferative in response to IL-4 and have greater survival when cultured with IL-4, in comparison to parental cells. In contrast, cells expressing a catalytically inactive form of SHIP have the exact opposite phenotype. These results are the first indication that the catalytic activity of SHIP can act as a positive mediator of growth and survival in cytokine signaling.

Introduction:

Cytoplasmic phosphatases are important regulators of signals that emanate from cell surface receptors. One of these phosphatases is SHIP, an SH2-containing inositol 5-phosphatase that can remove the 5-phosphate from PtdIns-3,4,5-P_3 or Ins-1,3,4,5-P_4. SHIP is composed of a N-terminal SH2 domain, a central phosphatase domain, two consensus NPXY sites which can bind proteins with PTB domains, and several interspersed COOH-terminal proline-rich regions (1-3). SHIP is tyrosine phosphorylated by a number of stimuli including, antigen specific B cell receptor(BCR) stimulation alone or FcγRIIb-BCR co-cross-linking as well as the cytokines IL-3, IL-2, GM-CSF, and steel factor (3-10). These same stimuli also induce the association of SHIP with Shc (3-7, 9-12). Studies of IL-3 and FcγRIIb signaling suggest that SHIP interacts with Shc via a bidentate interaction such that the SHIP SH2 domain binds to Shc Tyr (P)317 and the Shc PTB domain binds to the NPXY motif of SHIP (12, 13). Furthermore, studies also suggest that complexes of Grb2-Shc or SHIP-Shc are mutually exclusive since the Grb2 SH2 competes for the same site on Shc that SHIP binds. The functional importance of tyrosine phosphorylation of SHIP remains unclear. Although it is possible that SHIP phosphorylation affects its intrinsic catalytic activity (14), this remains unclear (2). Alternatively, it has been proposed that tyrosine phosphorylation of SHIP may modify its localization and thereby the availability of substrate and interacting proteins (2, 3). Importantly, the tyrosine kinase(s) responsible for cytokine inducible SHIP phosphorylation have not been identified.

The molecular basis by which SHIP modulates biological responses is likely to vary according to the signaling pathway examined. The SHIP protein has been most extensively studied in the context of FcγRIIb signaling. SHIP was initially isolated through its recruitment to the immuno receptor tyrosine based-based inhibitory motif (ITIM) of the receptor as well as through its association with Shc (6, 15, 16). SHIP has been shown to be important in the inhibition of B cell proliferation that occurs during cross-linking of FcγRIIb-BCR (17). This effect is postulated to be mediated by SHIP's ability to compete with the Grb2-Sos complex for binding to Shc, resulting in a block of Ras signaling pathway (9, 13). In contrast, analysis of the Ca^{2+} mobilization suggests that the enzymatic activity of SHIP may be involved in this pathway. SHIP recruitment to FcγRIIb inhibits BCR induced Ca^{2+} mobilization, which has been implicated in a diverse set of B cell events (6, 18). One proposed mechanism to understand SHIP's catalytic role in Ca^{2+} mobilization is based on the ability of SHIP to regulate pools of inositol lipids. Levels of PtdIns-3,4,5-P_3 are the critical regulators of Ca^{2+} signaling at least in part by their ability to support the activation of Tec family kinases (19, 20). This effect is mediated via the pleckstrin homology PH domains of Tec kinases and PLCγ, which preferentially bind PtdIns-3,4,5-P_3 and lead to the increased membrane targeting of these enzymes (19, 20). This in turn leads to the increased phosphorylation of PLCγ2 and subsequent Ins-1,4,5-P_3 production (19, 20). SHIP may inhibit membrane targeting and thereby activation of Tec kinases, preventing Ins-1,4,5-P_3 production, and subsequent Ca^{2+} mobilization by converting PtdIns-3,4,5-P_3 to PtdIns-3,4,-P_2. The resulting

increase in PtdIns-3,4,-P_2 products, may in turn increase the membrane targeting of other proteins containing PH domains that instead preferentially bind PtdIns-3,4,-P_2 (19-21).

Although SHIP is phosphorylated by many cytokines, the role of SHIP in cytokine signaling remains poorly understood. Initial reports suggested that SHIP plays a negative regulatory role in cytokine signaling (2, 22). Elucidation of SHIP's role *in vivo* has been aided by the recent report of the SHIP homozygous null mice (23). Consistent with the previous results with respect to IL-3 and GM-CSF, hematopoietic stem cells from SHIP (-/-) mice display increased colony formation in response to IL-3, GM-CSF, G-CSF, and steel factor, suggesting that SHIP, may in fact, act as a negative regulator of either proliferation and/or survival in response to these particular cytokines. Interestingly, the SHIP(-/-) mice display decreased survival secondary to lung infiltration from cells of the myeloid and granulocytic compartment correlating with increased colony formation observed in response to this subset of cytokines. Conversely, analysis of other cellular compartments suggests that SHIP may not solely act as a negative regulator, but potentially may serve other functions. In support of this notion, the null mice exhibit decreased bone marrow cellularity and a substantial reduction in pre-B cells with a concomitant reduction in peripheral B cells (23). It is unclear, whether the presumed defect in transition from the pro-B to pre-B cell stage is the result of failure to survive and/or inability to proliferate. However, these observations do suggest a potential positive role for SHIP in some instances, including B cell development.

Importantly, the mechanism underlying the action of SHIP in cytokine signaling remains to be determined, including the contribution of the many domains within SHIP. In particular, the role of the catalytic activity of SHIP in the regulation of cytokine signaling remains unknown. IL-4 induces SHIP tyrosine phosphorylation and recruitment to the IL-4R α chain (Pan et.al. submitted, and (24)). In this report, we investigated the role of SHIP in IL-4 signaling. Our studies show that SHIP can interact with additional members of the activated IL-4 signaling complex in addition to the receptor, including Shc and IRS-2. Further, co-expression of Jak1 and SHIP lead to their physical association and SHIP tyrosine phosphorylation. Finally, we demonstrate a role for the catalytic activity of SHIP in both proliferation and protection from apoptosis induced by IL-4.

Materials and Methods

Cells and culture conditions

32D myeloid cells stably expressing IRS-2 (32D/IRS-2, kindly provided by Dr. J. Pierce, NIH, Betheseda, MD) were maintained in RPMI-1640 supplemented with 10% FCS (Sigma Chem. Co., St. Louis, MO), 2mM L-glutamine, 50 μM β-mercaptoethanol(Bio-Rad, Hercules, CA), and 5% WEHI-3 conditioned medium as a source of IL-3. COS cells were maintained in DMEM containing 10%FCS and 2mM L-glutamine.

Plasmid construction

The cDNAs encoding murine wildtype SHIP, mSHIP(WT), and the murine catalytic mutant, m SHIP(D675C), inserted into the Bluescript II KS+ plasmid (Stratagene, Inc.). An Xba I-Hind III fragment from the BSKS+mSHIP(WT) and BSKS+mSHIP(D672A) encoding the open reading frame was blunted and ligated into a blunted HindIII site of the retroviral vector pMSCV-IRES-GFP (a kind gift of Dr. Gary Nolan) upstream of the IRES element. The vectors pBJ3-mJak1 and pBJ3-mJak3, which encode the cDNAs of, full length wildtype murine Jak1 and murine Jak3 are described elsewhere (25).

Transient transfections, retroviral transduction and establishment of cell lines

Transient transfection of COS cells was performed using lipofectamine (GICO-BRL). Briefly, 10^5 cells were plated on 35-cm dishes and transfected with 2 μg DNA as indicated in figure legend. Two days after transfection, cells were moved into medium of DMEM with 0.5% FCS. Retroviruses of pMSCV-IRES-GFP vector alone or those carrying SHIP cDNAs were packaged with the ΦNX-Eco cell line by calcium phosphate transfection and supernatant containing virions were harvested post transfection. Viral stocks were stored at –80°C or used immediately for infection after checking for transfection efficiency by GFP FACS analysis. 32D IRS-2 cells were infected as described (www-leland.stanford.edu/group/nolan). Infected cell cultures were expanded for 2-4 days prior to sorting. To identify stable cells expressing the gene of interest, cells were sorted for GFP by FACS analysis (FACStar, Becton Dickinson, Mountain View, CA) . Protein expression was confirmed by Western blot analysis of cells after sorting.

Cell stimulation, cell lysis, immunoprecipitation, and immunoblotting

32D IRS-2 cells were washed and resuspended in starvation medium containing RPMI-1640 medium, 2mM L-glutamine, 50 mM β-mercaptoethanol, and 0.5% FCS for 4 hrs at 37°C and then incubated in the presence or absence of murine IL-4 (10 ng/ml) or IL-3 (5% WEHI-3 conditioned medium). Cell lysate preparation was performed using methods previously described (26) for Shc, and SHIP immunoprecipitations, whereas for IRS-2 immunoprecipitation cell lysates were prepared as described (27). The immunopreciptation and/or immunoblotting of Shc, SHIP, Jak1, Jak3 and IRS-2 were performed as described (28). Antibodies against phosphotyrosine (4G10),

Shc, JAK3 and IRS-2 were obtained from Upstate Biotechnology, antibody against murine JAK1 from Santa Cruz, and polyclonal anti-SHIP antibody was previously described (9).

Proliferation Assays

32D IRS-2 cell lines were grown in RPMI-1640 and 5% WEHI-3 conditioned media 3 days prior to experiment. On the day of the experiment, cells were washed 3 times with complete RMP-1640 without 5% WEHI-3 conditioned media. Cells were cultured at a density of 2.5 X 10^4 cells per 200µl in a 96-well flat-bottom microtiter plate in culture media with varying concentrations of recombinant mIL-4 or 5% WEHI-3 conditioned medium in triplicate. Cells were pulse-labeled with 1µCi of [^{3}H]-thymidine (specific activity 6.7/Ci/mmol; NEN, Boston MA) for the last 8 hrs of the 48 hr culture and harvested. [^{3}H]-Thymidine incorporation was measured by a scintillation counter.

Apoptosis Analysis

32D IRS-2 cell lines were grown in RPMI-1640 and 5% WEHI-3 conditioned media for 3 days prior to experiment. Then, cells were washed 3 times with complete RMP-1640 without 5% WEHI-3 conditioned media. Cells were then stimulated with mIL-4 at 100ng/ml or 5%-WEHI-3 conditioned medium for a total of 24 hours. After 24 hrs of cytokine stimulation, cells were once again washed with complete RPMI-1640 medium without mIL-4 or WEHI-3. Subsequently, aliquots of cells were harvested at 0, 18, 24, 42, and 48 hrs. At each time point, Annexin-V-PE and PI staining was performed and analyzed by FACStar. The percentage of viable cells was calculated as the number of Annexin and PI double negative staining cells divided by the total number of cells analyzed (10,000).

RESULTS

Overexpression of SHIP increases IL-4 mediated proliferation

Our previous studies have shown that SHIP is recruited to the IL-4 receptor complex in cells cultured with IL-4 (Pan et. al. submitted). To examine the role of the catalytic function of SHIP in IL-4-mediated proliferation and protection from apoptosis, 32D/IRS-2 cells were established that overexpress either wildtype SHIP or a mutated SHIP called SHIP (D672A) in which the aspartate (D) at position 672 of phosphatase active site was mutated to alanine (A). This mutation rendered the protein catalytically inactive ((29) and data not shown). In order to establish these cell lines, SHIP cDNA constructs were transduced into cells via a bicistronic retroviral vector carrying GFP, to both select and monitor expression of SHIP by GFP expression (30). Using this method, we were able to establish cells overexpressing the wildtype (WT) SHIP and SHIP (D675C) proteins at levels 4 to 5-fold respectively over control cells infected with retroviral vector expressing GFP alone (Figure 1B).

To examine the role of SHIP in regulating IL-4 induced proliferation, transduced cells (which also express IRS-2) were starved of IL-3 and cultured with varying amounts of IL-4 for 48 hours. Proliferation was examined by ^{3}H-thymidine incorporation. Cells overexpressing wildtype SHIP exhibited greater proliferation in response to IL-4 stimulation in comparison to cells expressing the vector alone over a wide range of concentrations (Figure 1A). This effect was most pronounced at the lower IL-4 concentrations. For example, at 1 ng/ml, cells expressing SHIP (WT) incorporated as much as 15 fold more thymidine than cells expressing vector alone. In striking contrast, in cells expressing SHIP (D672A) the proliferative response to IL-4 was suppressed below the levels of control cells (Figure 1A). The finding that the catalytically inactive SHIP (D672A) suppresses proliferation suggests that the increased proliferation observed in cells overexpressing the wild type SHIP is not simply due to the disruption of protein-protein interactions mediated by SHIP overexpression.

To determine if SHIP also affected the growth of these cells in response to IL-3, thymidine incorporation was measured in these same cell lines grown in IL-3. A previous study has shown that over the first 72 hours of culture with IL-3, overexpression of wild type SHIP in DA-ER cells had no effect on cell number (13). In agreement with this report, there was no effect on the proliferation of 32D/IRS-2 cells overexpressing of SHIP (WT) at the concentration measured (Figure 1A). This result indicates that the positive role for the catalytic activity of SHIP in IL-4 –mediated proliferation is specific. These observations provide the first evidence that the catalytic activity of SHIP is not always an inhibitor of cytokine signaling *in vivo*, but rather may act as a positive effector as demonstrated for IL-4 mediated proliferation.

Overexpression of SHIP increases IL-4 mediated protection from apoptosis

IL-4 not only induces cellular proliferation, but can also protect cells from apoptosis (31-33). We therefore sought to determine if SHIP could alter the cell survival signals induced by IL-4. 32D/IRS-2 cells expressing SHIP (WT), SHIP (D672A), or the GFP vector alone were starved from IL-3 for 24hrs and then cultured with IL-4 for the next 24hrs. The duration of IL-4 mediated protection from apoptosis was measured after IL-4 withdrawal. This approach was taken because it may be more reflective of SHIP's effect on inositol lipid half-life, rather than continuous IL-4 treatment, which induces PtdIns-3,4,5-P_3 production (34).

Upon withdrawal from culture with IL-4, substantial differences in the number of viable cells existed when the cells overexpressing the SHIP (WT) and those with SHIP (D672A) were compared (Figure 2A). At 48 hours, the cultures of cells expressing wild type SHIP contained 28% viable cells, while the cultures of cells expressing SHIP (D672A) had only 5% viable cells. The cells expressing the GFP vector alone had an intermediate level of cell viability. The trend in differences in cell survival were consistent throughout the period measured. In addition to Annexin V staining and PI staining, FACS analysis of GFP expression was performed. Previously, it had been demonstrated that in cells expressing GFP vectors, GFP expression could be used as an index of apoptosis (e.g. (35)). A greater number of cells expressing SHIP(WT) remained GFP bright compared to SHIP (D672A) expressing cells at 42 hours (Figure 2C). In contrast, there was no difference observed in terms of the kinetics of survival in response to IL-3 among the cell lines expressing GFP alone, SHIP(WT), and SHIP (D672A) (Figure 2B).

In order to determine if the difference in cell viability observed in these IL-4 cultures was simply the result of increased proliferation, rather than protection from apoptosis, proliferation was measured in these cultures. Significantly, after 48 hrs of IL-4 withdrawal, all the cell lines had essentially no measurable thymidine incorporation (data not shown). This indicates that the increased number of viable cells observed in the cultures of cells overexpressing SHIP (WT) was not the result of the hyperproliferative phenotype observed above. These results strongly suggest that SHIP can increase IL-4 mediated protection from apoptosis independent of its role in IL-4-mediated proliferation and further that this function requires its catalytic activity.

IL-4 induces the association of SHIP with IRS-2 and Shc

Signaling pathways emanating from distinct regions of the IL-4R have been implicated in activation of gene expression and stimulation of proliferative responses to IL-4. Following ligand induced receptor oligomerization, the Jak1 kinase becomes activated and subsequently phosphorylates the IL-4Rα on tyrosine residues within its cytoplasmic domain, leading to

recruitment of downstream signaling effector molecules including Stat6 and IRS-1 or IRS-2 (36-38). The presence of either IRS-1 or IRS-2 is required for IL-4 stimulated proliferation (27, 39). These proteins, which contain both PH and PTB domains, are recruited to the activated human IL-4R by interaction with the phosphorylated tyrosine residue (Y497) (38, 40-42). Furthermore, this tyrosine has recently been implicated in the recruitment and phosphorylation, in response to IL-4 stimulation, of two related ras-GAP interacting proteins FRIP ($p56^{dok}$) and $p62^{dok}$, which contain PTB domains homologous to IRS proteins (43). In addition, Shc has been shown to bind this conserved tyrosine motif in the IL-4R (41). Several cytokines, including IL-3, have been reported to induce the association of Shc with SHIP (3, 7, 10, 11, 44, 45). We therefore sought to determine if this interaction could also be induced upon IL-4 signaling. Furthermore, given its importance for IL-4 induced proliferation, the requirement of IRS-2 for this interaction was determined.

The ability of IL-4 to induce the association of Shc with SHIP was explored in the 32D cell line. Parental 32D cells, which lack expression of IRS-1 and IRS-2, were compared to 32D cells stably expressing IRS-2. Protein extracts were prepared from these cells grown in the presence of either IL-4 or IL-3. Shc immunoprecipitates were examined by immunoblotting with the anti-phosphotyrosine antibody 4G10 (Figure 3A). Both IL-3 and IL-4 induced the tyrosine phosphorylation of the p52 Shc isoform. IL-3 also induced the tyrosine phosphorylation of the p46 isoform of Shc. Shc phosphorylation in response to IL-4 occurred in cells independent of the presence of IRS-2. These immunoblots also demonstrate a 145kD tyrosine-phosphorylated protein in Shc immunoprecipitates from cells grown in either IL-3 or IL-4. Reprobing these blots with antisera for SHIP demonstrated that this protein was SHIP (Figure 3A, lower panel). These results demonstrate that IL-4 can induce association of SHIP with Shc independent of IRS-2.

The activation of cellular proliferation in response to IL-4 has been proposed to occur through the interaction of phosphorylated IRS-1/2 molecules with additional SH2 domain signaling molecules such as the p85 subunit of PI-3 kinase or Grb2 (46-48). Because of the effects of SHIP on IL-4 mediated proliferation, we examined whether IL-4 could induce the association of SHIP with IRS-2. IRS-2 was immunoprecipitated from extracts of 32D +/- IRS-2 cells unstimulated or stimulated with murine IL-4. Immunoprecipitates were fractionated on SDS-PAGE and immunoblotted with anti-SHIP. IL-4 induced the association of SHIP with IRS-2 and not in control parental 32D cells lacking IRS-2 (Figure 3B). These data suggest that IL-4 induces the association of SHIP and IRS-2. The results taken together suggest that SHIP can associate with both IRS-2 and Shc in response to IL-4.

As demonstrated above, IL-4 induces the tyrosine phosphorylation of SHIP in cell that do not either express IRS-1 or IRS-2. To determine if the presence of IRS-1/2 can effect the kinetics of SHIP phosphorylation, 32D cells were grown in IL-4, and SHIP tyrosine phosphorylation examined over time. 32D cells exhibit a transient induction of SHIP phosphorylation peaking at

10 minutes and returning to basal levels by 30 minutes of IL-4 culture (Fig 3C). 32D cells that express IRS-2 also exhibited IL-4 induced SHIP phosphorylation but with altered kinetics with phosphorylation detected as early as 5 minutes. These results confirm that IRS-2 is dispensable for IL-4 induced SHIP phosphorylation. However, the kinetics of SHIP phosphorylation is altered by the expression of IRS-2.

The role of Jak1 and Jak3 in SHIP phosphorylation

The kinase(s) responsible for phosphorylation of SHIP in response cytokines has not been identified. In the case of FcγRIIb-BCR cross-linking Syk has been implicated as a kinase that potentially can phosphorylate SHIP (49). In addition, Lck has been implicated in the phosphorylation of SHIP in response to T cell receptor signaling. (50). Two kinases of the JAK family are activated by IL-4 in hematopoietic cells. Jak1 constitutively associates with the IL-4R and is both necessary and sufficient for IL-4 induced Stat6 and IRS-2 phosphorylation. Jak3 associates with the common γ chain (γC) and also becomes activated upon IL-4 stimulation presumably through transphosphorylation by Jak1 after receptor oligomerization. The induction of SHIP phosphorylation in response to IL-4 suggests that kinases activated by IL-4 may be capable of phosphorylating SHIP .

To further examine the mechanisms by which SHIP is phosphorylated and recruited to the IL-4 signaling complex, we examined whether SHIP can associate with either of these kinases (Figure 4). COS cells were transfected with an expression vector for SHIP, together with either Jak1 or Jak3 expression vectors. Cell lysates were then subjected to immunoprecipitation with anti-SHIP antibody, which was followed by immunoblotting with the anti-phosphotyrosine antibody, 4G10. Overexpression of SHIP alone did not lead to the presence of any tyrosine phosphorylated species. Strikingly, expression of SHIP with Jak1, but not Jak3, resulted in a heavily tyrosine phosphorylated species of 145kd, which was confirmed to be SHIP by reprobing with an antiserum to SHIP (Figure 4A). Consistent with the above result, Jak1, but not Jak3 co-immunoprecipitated with SHIP in these extracts (Figure 4B). The interaction of JAK1 with SHIP occurred without the addition of cytokine to the cultures.

To extend these data, the association of endogenous SHIP with Jak1 and Jak3 was examined. In order to remove the confounding factor of the IRS-2-SHIP association, the potential interaction of SHIP with either Jak1 and/or Jak3 was analyzed in the parental 32D cell line, lacking expression of IRS-1 and IRS-2 (Figure 4C). Interestingly, SHIP was found to immunoprecipitate with Jak1 in these cells. As observed in the COS cells experiments, association of SHIP with Jak1 occurred in cells without the addition of cytokine. Intriguingly, SHIP was also found to co-immunoprecipitate with Jak3 in 32D cells. This association was increased when the cells were cultured with IL-4. These data suggest that JAK kinases may be involved in the recruitment and/or phosphorylation of SHIP.

Discussion

IL-4 is a pleiotropic cytokine, which behaves as a critical regulator of a diverse set of processes within the immune system . Using a well-established *in vitro* model system to study the effects of IL-4 stimulation on immune cells, our data suggests that the catalytic function of SHIP serves as a positive regulator of certain IL-4 functions. The data demonstrate that overexpression of wildtype SHIP leads to hyperproliferation in response to a range of IL-4 concentrations. In striking contrast, a catalytic inactive mutant form of SHIP (D672A) suppresses IL-4 induced proliferation. Significantly, the catalytic activity of SHIP also behaves as a positive regulator of IL-4 mediated protection from apoptosis. It is interesting that overexpression of SHIP does not effect IL-3 induced proliferation or IL-3 mediated protection from apoptosis under the conditions examined. The reason for the observed differences between IL-4 and IL-3 in the 32D cells examined here is unclear. Prior studies of SHIP (-/-) mice suggest that SHIP is an inhibitor of IL-3 function (23). Therefore, one possibility is that because the 32D cells examined are IL-3 dependent we may have selected against SHIP functional activation by IL-3 in these stably transfected cell lines. However, the retained ability of IL-3 to induce SHIP phosphorylation and Shc association argues against this possibility (Figure 3A, data not shown). In addition, a previous report has demonstrated that ectopic overexpression of SHIP(WT) in IL-3 dependent cells does not affect cell growth over at least 72 hours of culture (50). An alternative possibility is that IL-3 and IL-4 may utilize different mechanisms to protect cells from apoptosis (51). In support of this hypothesis, a recent study demonstrates that although both IL-3 and IL-4 can lead to AKT activation, only IL-3 induces Bad serine phosphorylation. (51). Therefore, it may be that SHIP differentially affects the divergent pathways by which IL-4 and IL-3 protect cells from undergoing apoptosis.

It remains to be fully determined which domains of SHIP are involved in the regulation of signaling activated by different cytokines. It is reasonable to postulate that different domains of SHIP may act in concert or antagonistically to integrate cytokine responsiveness. Perhaps, the catalytic function may have no impact on signaling depending on the cytokine or pathway. Thus, it cannot be excluded that the catalytic activity of SHIP cooperates with other domains to mediate the IL-4 mediated pathways examined. Similarly, the domains involved in the negative regulation of IL-3 signaling cannot be distinguished in the context of the SHIP (-/-) mouse. Therefore, the functional effects of SHIP may vary by domain, cell type, cytokine examined, or by the biological endpoint measured.

Many cytokines induce SHIP phosphorylation, yet the tyrosine kinases involved have not been identified. In IL-4 signal transduction, Jak1 is necessary to phosphorylate the two previously recognized downstream signaling effectors, Stat6 and IRS-1/2 (52, 53). Analysis of JAK-SHIP interactions in COS cells suggests that Jak1, but not Jak3, may mediate SHIP phosphorylation

independent of cytokine stimulation. Importantly, endogenous Jak1 was also shown to constitutively associate with SHIP in 32D cells. In contrast, endogenous Jak3 inducibly associates with SHIP upon IL-4 stimulation. The reason for the disparity in Jak3 and SHIP interaction in COS versus 32D cells remains to be determined, but may stem from a requirement for an intermediate scaffolding protein such as the common γc chain not present in COS cells. However, these experiments have not formally proven that SHIP is a JAK1 substrate *in vivo*. Other experiments suggest that unidentified kinases phosphorylate SHIP *in vivo* in the absence of IL-4 (data not shown). However, to our knowledge, Jak1 is the first candidate kinase identified capable of mediating SHIP tyrosine phosphorylation in a heterologous system.

In vivo, the stoichiometry and spatial association of SHIP with members of the activated IL-4 signaling machinery is likely to be complex. Jak1 is presumed to associate with the membrane proximal region of the cytoplasmic domain of the IL-4 receptor α-chain (IL-4R). At the opposite end of the receptor cytoplasmic tail, we have identified a conserved Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) consensus (Pan et. al., submitted). Cells expressing an IL-4Rα chain mutated in the ITIM motif have increased proliferative response to IL-4. *In vitro*, an IL-4Rα chain derived ITIM peptide binds not only SHIP, but also SHP-1 and SHP-2. Furthermore, both SHP-1 and SHIP co-immmunoprecipitate with the IL-4Rα chain upon IL-4 treatment ((Pan et. al. submitted) and (26)). At first glance, these two results, the possible recruitment of SHIP to a functional ITIM in the IL-4Rα chain and a positive role for SHIP in proliferation and apoptosis appear at odds. However, there are several explanations for these seemingly conflicting results. One possibility is that although the ITIM in the IL-4Rα chain can bind SHIP, the effects seen in cells expressing an IL-4Rα chain lacking this motif result not from loss of SHIP binding, but through the loss of recruitment of SHP-1 and/or SHP-2 or as yet other unidentified proteins. A corollary to this argument is that SHIP maybe recruited to the IL-4R complex via other mechanisms besides the ITIM. Indeed, the biochemical data in this report suggest that SHIP binds directly or indirectly to multiple signaling partners including IRS-2, Jak1 and Shc. The finding that cells expressing an IL-4Rα chain ITIM mutant are still capable of phosphorylating SHIP supports a model whereby the IL-4Rα chain ITIM may not be the only site of SHIP recruitment to the activated IL-4 receptor complex (24). Alternatively, SHIP may perform different functions if recruited to distinct portions of the receptor complex. Such differences could arise from the availability of different substrates to SHIP catalytic activity, secondary to either spatial or temporal differences.

Once recruited to the IL-4 receptor complex, the downstream targets affected by SHIP activity modulating protection from apoptosis and proliferation remain to be determined. The positive role that SHIP plays in IL-4 signaling suggests that by altering inositol phosphate composition at the membrane SHIP either enhances the recruitment of proteins that potentiatesIL-4 induced proliferation, or decreases the recruitment of proteins that inhibit proliferation. For example, the products of SHIP metabolism may cause increased membrane targeting of PH-containing

IRS-1 and IRS-2 resulting in increased proliferation. In fact, recent data indicates the PH domain of IRS-1 is important for IRS-1 mediated protection from apoptosis (54). However, our preliminary results demonstrate no difference in the level of IRS-2 tyrosine phosphorylation induced by IL-4 in cells expressing the wild type of catalytic inactive forms of SHIP. Another possibility is that SHIP affects the recruitment or activation of other PH containing proteins in response to IL-4. Candidates for such proteins include the PH-containing rasGAP interacting proteins FRIP ($p56^{dok}$) and $p62^{dok}$ recently implicated in IL-4 signaling (43). Although the functions of dok proteins have not been defined, the finding that p62dok is phosphorylated by the BCR-Abl oncoprotein suggests it may act in cellular proliferation (55-57). Although inositol phosphate composition of the membrane can alter the recruitment of PH-containing proteins, it is believed that the recruitment/activation of proteins containing other structural motifs may also be sensitive to inositol phosphate composition (58). Therefore, the mechanism of SHIP regulation of IL-4 induced signals may involve yet to be identified proteins.

A positive signaling role for SHIP has been suggested by the defects in B cell development found in SHIP (-/-) mice and by the association of SHIP with the BCR-ABL oncoprotein. Our finding that overexpression of wild type SHIP can increase the proliferation of cells in response to IL-4 is the most direct evidence that SHIP can be a positive signaling molecule. Understanding the mechanism by which SHIP increases proliferation and cell survival may provide important insights into the role of inositol phosphates in signaling activation and perhaps cellular transformation.

Figure Legends

Figure 1

Overexpression of SHIP enhances IL-4-induced proliferation. (A) 32D IRS-2 cells infected with retroviruses containing GFP alone (GFP), SHIP(WT) and catalytic mutant SHIP(D672A) were incubated with various doses of mIL-4 as indicated or 5%-WEHI-3B conditioned media for 48hrs as described in Material and Methods. Proliferation was measured in triplicate wells by [^{3}H] thymidine uptake. One of three representative experiments is shown. (B) The expression level of SHIP in the cells transduced with SHIP(WT), SHIP(D672A) and GFP vector alone was determined by a Western blot analysis with anti-SHIP polyclonal antibody. Control cells do not express SHIP.

Figure 2

Overexpression of SHIP enhances IL-4-mediated protection from apoptosis. 32D IRS-2 cells transduced with GFP vector alone, SHIP(WT) and catalytic mutant SHIP(D672A) were passaged three days prior to experiment, washed from complete medium and incubated either with (A) IL-4 (100 ng/ml) or (B) IL-3 (5% WEHI-3B) for 24hrs. Subsequently, cells were withdrawn from cytokine and aliquots of cells were analyzed for viability at the time indicated by annexin-PE and PI staining. FACS analysis of 10^4 cells was performed for each sample and percent non-apoptotic cells calculated as described in Material and Methods. One of three representative experiments is shown. (C) GFP expression as another index of viability was performed at each time point indicated in (A) by FACS analysis at 48hrs.

Figure 3

Phosphorylation and association of SHIP with components of the IL-4 receptor complex. (A) Requirement for IRS-2 in IL-4 induced association of SHIP association with Shc. Parental 32D cells or cells stably transfected with IRS-2 were serum and factor starved for 4 hrs and then stimulated for 15 minutes with mIL-4 (100 ng/ml) or IL-3 (5% WEHI-3B) conditioned medium. Shc was immunoprecipitated from these cells, and the immunoprecipitates fractionated by SDS-PAGE and subjected to immunoblotting with anti-phosphotyrosine antibody (p-Tyr). Subsequently the blot was reprobed with anti-SHIP and then anti-Shc antibodies as seen in the lower panels. (B) Association of SHIP with IRS-2. Cell lysates were prepared from 32D cells -/+ IRS2 as described in (A) and immunoprecipitated with anti-IRS-2 antibody. Immunoprecipitates were fractionated by SDS-PAGE and subjected to immunoblotting with anti-SHIP antibody (upper panel) and subsequently reprobed with anti-IRS-2 antibody (lower panel). (C) Time course of IL-4 induced phosphorylation of SHIP in parental 32D cells versus cells stably transfected with IRS-2. 32D cells +/-IRS-2 were stimulated with mIL-4 for indicated times and cell lysates were

subjected to immunoprecipitation (IP) with anti-SHIP and analyzed by immunoblotting with anti-phosphotyrosine (p-Tyr) and anti-SHIP antisera.

Figure 4

SHIP interaction with JAK kinases. (A) Jak1 involvement in SHIP phosphorylation. COS cells were transfected with the cDNAs as indicated and starved in 0.5% FCS for 48hrs. SHIP was immunoprecipitated from these cells, and the immunoprecipitates subjected to immunoblotting with anti-phosphotyrosine antibody (p-Tyr) and subsequently reprobed with anti-SHIP antibody (lower panel). (B) Jak1 associates with SHIP. Cell lysates from COS cells transfected with various cDNAs were prepared as above in (A) and immunoprecipitated with anti-SHIP antibody. The immunoprecipitates were separated by SDS-PAGE and then immunoblotted simultaneously with a combination of anti-Jak1 and anti-Jak3 antibody followed by reprobing with anti-SHIP antibody. (C) SHIP associates with endogenous JAKs. Parental 32D cells were serum and factor deprived for 4 hrs and then stimulated (+) or not (-) for 15 minutes with mIL-4 (100 ng/ml). Jak1 or Jak3 were immunoprecipitated from these cells, and the immunoprecipitates fractionated on SDS-PAGE and immunoblotted with anti-SHIP antibody. The blots were subsequently reprobed with antibodies to Jak1 and Jak3. Non-immune serum (NRS) was used as control.

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Analysis of cytokine signaling in patients with extrinsic asthma and hyperimmunoglobulin E

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Background: Recent data suggest that the regulation of class switching to IgE by cytokines is mediated by STAT transcription factors. The induction of IgE by IL-4 and IL-13 occurs through the activation of the intracellular signal-transducing protein Stat6, whereas the inhibition of IgE class switching by interferon-γ (IFN-γ) occurs through the activation of Stat1.
Objective: We hypothesized that in extrinsic asthma or in cases of markedly elevated IgE (ie, hyperimmunoglobulin E [HIE]) increased levels of IgE may be associated with alterations in the cytokine levels or the activation of Stat6.
Methods: PBMCs and sera from 8 patients with extrinsic asthma (mean IgE, 285 ± 100 IU/mL), 3 patients with HIE (mean IgE, 7050 ± 1122 IU/mL), and 14 nonatopic control subjects (mean IgE, 112 ± 28 IU/mL) were analyzed.
Results: The mean IL-4 level detected by ELISA was much greater in patients with HIE than control subjects (88.6 ± 11.5 pg/mL vs 11.5 ± 7.1 pg/mL, $P = .005$), and increased IL-4 levels among patients with both asthma and HIE correlated with the increased IgE levels. In contrast, IL-13 levels were not elevated. Levels of Stat6 protein present in PBMCs did not differ in the patients and control subjects. Examination of Stat6 DNA-binding activity demonstrated no activation of IL-4 signaling in patients with either HIE or acute asthma. Interestingly, evidence for the presence of B cells that have already switched to IgE was seen in PBMCs of several patients with asthma or HIE.
Conclusion: These results indicate that (1) IgE production in asthma and HIE usually is associated with elevated levels of IL-4, but not IL-13, in the peripheral blood; (2) the increased sera IL-4 levels in asthma and HIE are not sufficient to induce Stat6 activation in PBMCs; and (3) evidence of switch recombination to ε may be detected in isolated cases of elevated IgE. This implies that high levels of IgE in these patients either results from B cells that have already undergone class switching, from Ig class switching that is localized to target tissues, or both. (J Allergy Clin Immunol 1998;102:503-11.)

Key words: IL-4, IL-13, hyperimmunoglobulin E, extrinsic asthma, Stat6, switch recombination

Abbreviations used

EMSA:	Electrophoretic mobility shift assay
GAS:	γ-Activating site
HIE:	Hyperimmunoglobulin E
HIES:	Hyperimmunoglobulin E syndrome
IRF-1:	Interferon response factor-1
Jak:	Janus kinase
NS:	Nonsignificant

The T_{H2} cytokine IL-4 has been shown to be a key mediator in Ig class switching to IgE by B cells.[1,2] The requirement of IL-4 for switching to IgE in mice has also been confirmed in vivo. For example, IgE levels in mice homozygous for a mutation in the IL-4 gene are undetectable or strongly reduced, even after infection with the nematode *Nippostrongylus brasiliensis*.[3,4] More recently, a second cytokine secreted by human T_{H2} cells, IL-13, has been shown to share biologic function with IL-4, including induction of IgE synthesis.[5-7] The effects of these T_{H2} cytokines can be inhibited by the T_{H1} cytokine IFN-γ, which has been shown to nearly abrogate IL-4–mediated induction of IgE by B cells.[1,2] The actions of these cytokines appear to result from their modulation of germline-ε transcription, a requisite step before class switching to IgE. These observations have prompted a model in which T_{H1} and T_{H2} cells mediate class switching to IgE through cytokines that antagonistically regulate transcriptional activity at the germline-ε promoter.[8,9]

The regulation of transcription by the cytokines IL-4, IL-13, and IFN-γ occurs through the intracellular signaling pathway termed JAK-STAT pathway.[10-14] In hematopoietic cells, IL-4 binds to a cell surface receptor on resting IgM+ B cells that consists of a heterodimer of the ligand-specific α-chain (IL-4Rα) and the common γ-chain (γC). Dimerization of the receptor leads to the activation, likely by transphosphorylation, of Janus kinase (Jak) 1, which is physically associated with IL-4Rα, and Jak3, which is associated with the γC chain.[15] The activation of these Jaks leads to the phosphorylation of several tyrosine residues contained within the cytoplasmic tail of IL-4Rα, which in turn serve as docking sites for the binding of the intracellular transducing protein Stat6 by means of interactions between one of several phosphotyrosyl motifs of the IL-4Rα and src homology domains of Stat6. Once recruited to the receptor complex, Stat6 becomes tyrosine phosphorylated, which allows it to form homodimers that translocate to the nucle-

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us and bind to IL-4 response elements that contain the sequence TTCN(4)GAA present in the promoters of IL-4–inducible genes, such as CD23 and Iε.[8,11,16-18] Because the IL-4Rα chain is a component of the IL-13 receptor,[19-20] the downstream signaling initiated by IL-13, including the activation of Stat6, overlaps with the signaling activated by IL-4.

Proof of the importance of Stat6 to cytokine-mediated signal transduction was provided from recently generated Stat6 knockout mice. These mice, which lacked expression of Stat6, are unable to initiate T_{H2} proliferative responses, increase CD23 and MHC class II expression on B cells in response to IL-4, or elicit a heightened IgE response after helminth infection or anti-IgD.[21-23] Stat6 knockout mice also are unable to induce MHC class II expression on peritoneal macrophages or decrease the nitric oxide production in response to IL-13.[24]

The production of high levels of IgE in response to antigen requires the occurrence of several events. The first key step appears to be the stimulation of T_H cells capable of producing type-2 cytokines. In the absence of type-1 cytokines, type-2 cytokines induce germline-ε transcription in IgM+ B cells.[9,16,17,25] Both IL-4 and IL-13 can induce these transcripts in human B cells.[5] Another key step, the deletional recombination event that is critical to Ig class switching, requires both germline-ε transcription and a B-cell activation signal after contact between the B-cell surface molecule CD40 and its ligand CD40L, which is expressed by activated T_H cells.[8,17,25-27] Subsequently, deletional recombination occurs between regions of repetitive DNA (switch [S] regions) present upstream from each heavy-chain locus, juxtaposing the fully assembled variable region (composed of rearranged VDJ gene segments) directly upstream of different heavy-chain genes that encode for a particular Ig isotype. The switched B cell then differentiates further into an antibody-secreting cell. For example, in the case of Ig class switching from IgM to IgE, the Cμ locus and downstream genes are deleted so that the Cε gene becomes juxtaposed directly 3' to the VDJ region, and VDJ-Cε transcripts are encoded.[8]

The identification of signaling molecules essential for specific class switching to IgE may enable further definition of the mechanisms underlying human disease states characterized by high levels of IgE. Hyperimmunoglobulin E syndrome (HIES) is a rare congenital immunodeficiency characterized by markedly elevated serum levels of IgE, characteristic facial features, chronic dermatitis, and lifelong recurrent severe infections.[28,29] Variants of this disease (ie, hyperimmunoglobulin E [HIE]) also have been described and are characterized by markedly elevated IgE in the absence of severe infections. Extrinsic asthma is a far more common disease characterized by increased serum levels of IgE. Our aim was to study the role of cytokine-induced intracellular signaling critical to IgE regulation in these 2 disorders, one characterized by symptoms localized to the airways and the other by extremely elevated levels of IgE. We hypothesized that some patients with acute exacerbations of extrinsic asthma or HIE may demonstrate measurable systemic alterations in the molecular regulation of IgE production. More specifically, we hypothesized that some subjects with extrinsic asthma or HIE may demonstrate increased systemic expression of T_{H2} cytokines in association with the activation of Stat6.

METHODS

Patient selection

Eight adult subjects with extrinsic asthma were recruited on presentation to the Presbyterian Hospital Emergency Department complaining of acute exacerbation of their asthma. All subjects with asthma met the clinical definition for asthma proposed by the American Thoracic Society.[30] Exclusion criteria included smoking, parenteral or oral corticosteroids taken within 4 weeks of entry into the study, and asthma or bronchospasm due to nonallergic causes. Three adult subjects with HIE were recruited, 2 from the Mt Sinai Hospital Immunology Clinic (New York) and 1 from a private referral. All 3 of these subjects were given diagnoses of variants of HIES characterized by markedly elevated IgE levels in the absence of severe lifelong systemic infections and the characteristic facial features. Nonatopic adults first seen at the Presbyterian Hospital Emergency Department or Clinic for other medical reasons served as control subjects. Institutional review board approval had been obtained, and all of their guidelines were followed.

Peripheral blood was drawn from all patients and nonatopic control subjects. Serum was separated by centrifugation immediately and stored at –70° for subsequent ELISA and RIA. PBMCs were isolated by Ficoll-Hypaque (Sigma Chemical Co, St. Louis, Mo) gradient as previously described.[31]

Cell culture and in vitro cytokine stimulation

Ramos 266 (human B cells) cells used as positive controls for several assays were grown in Iscove's modified Dulbecco's medium supplemented with 10% FCS (Sigma Chemical Co, St. Louis, Mo), 100 U/mL penicillin, and 100 μg/mL streptomycin and incubated at 37° C and 5% CO_2. Positive control Ramos cells or PBMCs were stimulated with human rIL-4 (gift from Dr Satwant Narula, Schering-Plough Corp., Kenilworth, NJ) for 15 minutes at a final concentration of 100 U/mL before generation of RNA or whole cell extracts. U266 (IgE-secreting myeloma) cells were grown in RPMI-1640 medium supplemented with 15% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and incubated as described above before extraction of DNA.

Generation of whole cell extracts

Whole cell extracts were prepared by using a detergent-free buffer as previously described.[32] Briefly, pelleted cells were incubated in RSB lysis buffer (5 mmol/L Tris [pH 7.4], 5 mmol/L NaCl, 3 mmol/L $MgCl_2$, 100 μmol/L Na_3VO_4, 1 mmol/L dithiothreitol, and 0.2 mmol/L phenylmethylsulfonyl fluoride) and rapidly resuspended in 30 mL of lysis buffer (Buffer C; 20% glycerol, 20 mmol/L Hepes [pH 7.9], 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 100 μmol/L Na_3VO_4, 3 μmol/L aprotonin, 1 μmol/L pepstatin, and 1 μmol/L leupeptin). The supernatant fraction (containing DNA-binding proteins) was removed by centrifugation and stored at –70° C.

Cytokine ELISAs

Human IL-4, IL-13, and IFN-γ sera levels were measured by using a commercially available ELISA kit (Immunotech, Inc., Westbrook, Me) according to the procedure indicated by the man-

ufacturer. Performance characteristics included a sensitivity of 5 pg/mL and a standard range of 0 to 1000 pg/mL of sera for IL-4, a sensitivity of 1.5 pg/mL and a standard range of 0 to 500 pg/mL for IL-13, and a sensitivity of 4 pg/mL and a standard range of 0 to 1250 pg/mL for IFN-γ. All specimens were analyzed in duplicate.

Western blot analysis

Five micrograms of whole cell extracts from subjects were resuspended in 1.3× SDS loading buffer (20% glycerol, 2% SDS, 0.025 mg/mL bromophenol blue, 0.125 mol/L Tris [pH 6.8], and 0.5% β-mercaptoethanol) and analyzed by SDS-PAGE (7% gel) as previously described.[33] Nitrocellulose membranes were blotted with an antibody to Stat6 (M-20; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) at 1:1000 dilution, analyzed after incubation with horseradish peroxidase–linked rabbit Ig secondary antibody (Amersham Corp., Arlington Heights, Ill), and detected with enhanced chemiluminescence (Amersham International plc, Buckinghamshire, England). The blots were then stripped and reprobed with an antibody to β-actin (Sigma Immunochemicals, St. Louis, Mo) at 1:1000 dilution.

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was performed as previously described.[11,34] Briefly, DNA binding was done in 40 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.1 mmol/L EGTA, 20 mmol/L HEPES (pH 7.9), 4% Ficoll, 0.5 mmol/L dithiothreitol, 1.2 mg/mL BSA, 200 μg/mL poly(dI-dC:dI-dC), 5 to 10 μg of whole cell extract, and 1 ng of probe. The P^{32}-labeled probe was to interferon response factor-1 (IRF-1) γ-activating site (GAS) element with the following sequence: 5' gatcGATTTCCCCGAAAT 3'. We have previously shown that several activated Stat proteins bind the GAS element.[34] The complexes were resolved on 0.22× Tris-buffered EDTA/4.5% acrylamide (29/1) gels.

DNA extraction, PCR, and Southern blot analysis

Total DNA was extracted from the whole cell extract pellet after incubation at 55° C for 12 to 24 hours in lysis buffer (50 mmol/L Tris HCl [pH 6.8], 5 mmol/L EDTA, 0.2% SDS, and 200 mmol/L NaCl) and 0.2 mg of proteinase K. Samples were then mixed with equal volume phenol and SEVAG (chloroform:isoamyl alcohol 24:1). The supernatant was precipitated in 100% ethanol and dissolved in TE buffer. Switch junction fragments (Sμ/Sε) were amplified from the DNA by means of nested PCR. The first round of PCR was performed with primers S6 (5'-CTGCAGACTCAGAAGGGAGGGGA TGCTCCG-3') and S4 (3'-ACTGATCCAAGA CAGGAGTGTGGCGGATGT-5') on 200 ng DNA, and the second round was performed on a 5-μL aliquot from the first round of PCR with primers S7 (5'-GAGGGTGGTAATGATTGGTAATGCTTTGGA-3') and S9 (3'-GAACCCTGAGG TCCGGTCCCCGCTTCCCGG-5') as previously described.[35,36] The PCR products were Southern blotted and incubated with a P^{32}-labeled probe derived from a 2.3 kb BAMHI fragment containing Sε.

RIA

Total human IgE was measured by a solid-phase immunoradiometric assay by using a commercial kit (Coat-A-Count Total IgE IRMA; Diagnostics Products, Corp, Los Angeles, Calif). The assay has a sensitivity of 0.5 IU/mL. All specimens were analyzed in duplicate.

Data analysis

Comparisons between mean sera levels of individual cytokines or IgE were made with Student's *t* test for means assuming equal variance. Regression analysis was performed to determine the degree of correlation between cytokine and IgE levels. Results are displayed as ± SE. A *P* value of less than .05 was considered statistically significant.

RESULTS

IgE determinations

To study the regulation of STATs in human patients with high IgE levels, we recruited patients with HIE and patients with extrinsic asthma. The 8 patients recruited with extrinsic asthma had mean IgE levels of 285 ± 100 IU/mL (range, 99 to 972 IU/mL). Three others initially recruited were excluded from further study because of IgE levels less than 99 IU/mL. The 3 patients referred for evaluation of HIE had mean IgE levels of 7050 ± 1122 IU/mL (range, 5828 to 9268 IU/mL). The 14 nonatopic control subjects had mean IgE levels of 112 ± 28 IU/mL (range, 5 to 364 IU/mL). These results confirm that our selection criteria provided subjects with abnormally elevated IgE levels that were increased over the levels of nonatopic control subjects.

Cytokine analysis

Most allergen-specific CD4+ T-cell clones derived from atopic patients' peripheral blood lymphocytes secrete elevated levels of IL-4 and IL-5.[37,28] Spontaneous levels of IL-4 and IL-5 also have been shown to be increased in the sera of subjects with allergic asthma.[39,40] In comparison, the number of CD4+ T-cell clones capable of producing IL-4 do not differ among patients with HIES, patients with atopic dermatitis, and healthy control subjects in one report,[41] nor does the extent of IL-4 production after in vitro stimulation of PBMCs with mitogens differ.[41,42] To characterize more precisely and to compare spontaneous sera IL-4 levels in extrinsic asthma, which is associated with primarily pulmonary symptoms and moderately elevated IgE levels, and in HIE, a variant of a systemic disorder that is associated with extremely elevated IgE levels but not necessarily target organ–related symptoms or severe infections, we measured sera IL-4 levels in all patients and nonatopic control subjects. Sera IL-4 levels are much greater in patients with HIE compared with control subjects (88.6 ± 50.4 pg/mL vs 11.5 ± 7.1 pg/mL, $P = .005$) (Fig. 1). There is a trend towards increased IL-4 levels among patients with asthma compared with control subjects that does not reach statistical significance (25.0 ± 7.7 pg/mL vs 11.5 ± 7.1 pg/mL, *P* = not significant [NS]). Among all patients, the elevated IL-4 levels correlate positively with increased IgE levels ($r = 0.69$). We conclude that sera IL-4 levels are markedly elevated in patients with HIE and that in both diseases these elevations correlate significantly with increased IgE levels.

IL-13, another T_{H2} cytokine, shares the function of regulating IgE class switching in B cells in vitro with IL-4.[5] Because IgE-mediated mechanisms are known to be involved in extrinsic asthma and HIE, then IL-13, like IL-4, may contribute to their pathogenesis. However, fewer data exist on the expression of IL-13 in peripheral

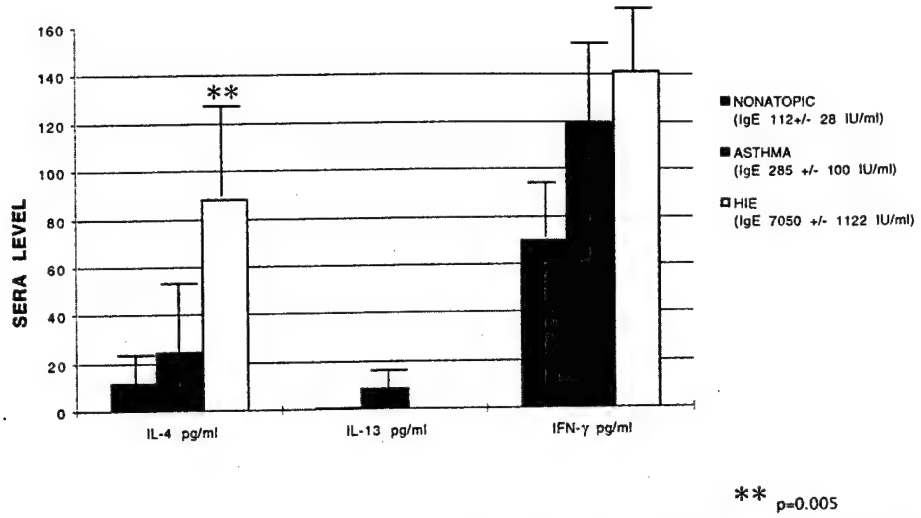

FIG 1. IL-4, IL-13, and IFN-γ expression during IgE-mediated disease. Sera cytokine levels were measured by ELISA. Data are shown as mean ± SE. IL-4 levels were significantly greater in patients with HIE than nonatopic control subjects, whereas IL-13 and IFN-γ levels did not differ among patients with HIE, asthma, or nonatopic control subjects. $**P = .005$.

blood during allergic inflammation, although one group has reported that sera levels were undetectable in patients with perennial allergic rhinitis.[43] To provide data on the systemic expression of IL-13 in other IgE-mediated diseases and to determine whether IL-13 levels correlate with IL-4 or IgE levels, sera IL-13 levels were measured in all patients and control subjects. The mean IL-13 level is 0.6 ± 0.6 pg/mL among nonatopic control subjects, 12.2 ± 12.2 pg/mL among patients with asthma, and 0.4 ± 0.4 pg/mL among patients with HIE and are not statistically different (Fig. 1). Only 1 subject with asthma had a markedly increased level of serum IL-13 (97.9 pg/mL vs 0.1 ± 0.1 pg/mL for the remaining patients). This patient has no detectable IL-4, a markedly elevated IFN-γ level of 530.5 pg/mL, and a minimally increased IgE level of 164 IU/mL. These data suggest that sera levels of IL-13, unlike IL-4, usually are not elevated in association with moderately increased IgE levels in extrinsic asthma or markedly increased IgE levels in HIE.

Most allergen-specific CD4+ T-cell clones derived from atopic patients' peripheral blood lymphocytes secrete no detectable or low amounts of IFN-γ.[37,38] In comparison, sera IFN-γ has been reported to be decreased,[44] nonelevated,[45,46] and paradoxically increased[45] in individuals with asthma exacerbations. In comparison, some investigators report that the production of IFN-γ by both T-cell clones and PBMCs from adults with HIES in response to phytohemagglutinin stimulation or galactose-oxidase induction is decreased.[41,47] In addition, IFN-γ inhibits IgE production by PBMCs both in vitro and in vivo in this disease,[48] and it has been proposed that defective production of IFN-γ is responsible for the overproduction of IgE in HIES.[41] However, the data are mixed as another group was unable to reproduce such findings or show that the secretion of IFN-γ decreases after induction with PMA and ionomycin.[42] To characterize further the expression of systemic IFN-γ during asthma first seen at the emergency department and in cases of HIE and to determine the relation between IFN-γ levels and IL4 and IgE levels, sera from all patients and control subjects were analyzed for IFN-γ. The mean IFN-γ levels were not significantly decreased in subjects with asthma (119 ± 65 pg/mL vs 70 ± 30 pg/mL among control subjects, P = NS) or in the 3 subjects with HIE (140 ± 25 pg/mL vs 70 ± 30 pg/mL among control subjects, P = NS). The IFN-γ levels do not correlate with the IL-4 levels or the IgE levels ($r = -0.1$ and 0.16, respectively). We conclude that in these 2 patient populations, despite the elevated IL-4 and IgE levels, there is no consistent pattern of expression of IFN-γ in the sera.

Stat6 protein levels

Stat6 has been shown to be essential for the induction of genes by IL-4 and IL-13. Furthermore, in transfection experiments the overexpression of Stat6 in certain cell lines leads to increased sensitivity to IL-4 and increased IL-4–mediated transcription.[49] These data suggest that the level of Stat6 protein may be the limiting factor for IL-4–induced transcription. Therefore one model for diseases associated with high IgE levels is that cells from these individuals express increased levels of Stat6, which would lead to a heightened response to IL-4 and increased class switching to IgE. To determine whether the level of expression of this protein is increased in PBMCs from subjects with IgE-mediated disease, levels of Stat6 were measured from whole cell extracts and compared among patients and control subjects. B-cell line Ramos and PBMCs stimulated with recombinant IL-4 in vitro served as positive controls for Stat6 expression, and protein levels from these whole

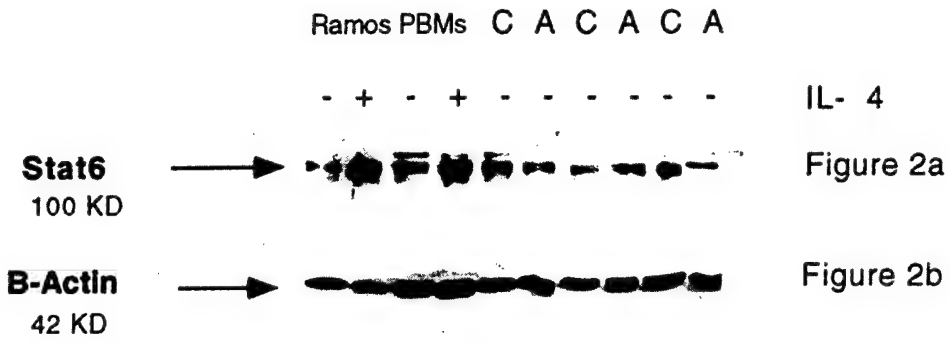

FIG 2. Western blot of whole cell extracts derived from PBMCs of 3 different representative subjects with asthma and simultaneously recruited nonatopic control subjects probed with α-Stat6 **(A)** and α-β-actin **(B)**. Ramos cells and PBMCs (nonatopic control subjects) were stimulated with recombinant human IL-4 (100 U/mL) for 15 minutes before generation of whole cell extracts. Although nonactivated Stat6 is present in all asthmatic subjects *(A)* and nonatopic control subjects *(C)* represented here and in all subjects not shown, levels are not increased in patients **(A)**. β-Actin levels are equivalent in all specimens **(B)**.

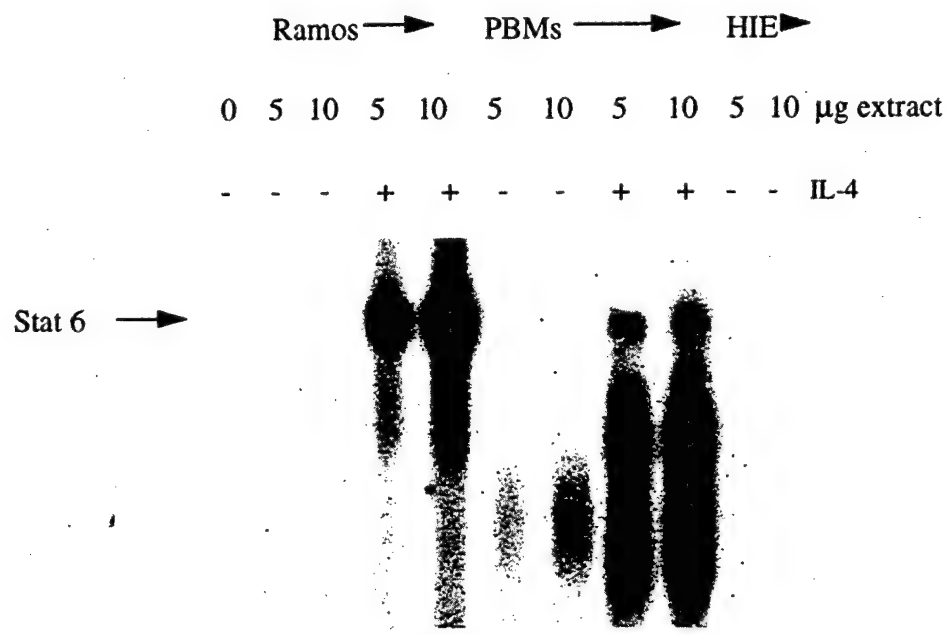

FIG 3. EMSA of representative patient with HIE. Whole cell extracts of PBMCs were probed with GAS element from IRF-1 gene. Ramos cells and PBMCs stimulated in vitro for 15 minutes with recombinant IL-4 (100 U/mL) served as positive controls for Stat6. Stat6 activation is not detected in this patient with HIE and markedly elevated IgE (9268 IU/mL) and IL-4 level (174.7 pg/mL) or in any other patients or nonatopic control subjects tested.

cell extracts were compared with those derived from patients with extrinsic asthma or HIE incubated with an antibody to Stat6. Stat6 was widely expressed in PBMCs derived from all tested individuals with extrinsic asthma and HIE and nonatopic control subjects (Fig. 2, *A*). The level of expression in patients with extrinsic asthma or HIE is not increased over that of control subjects. Probing of this Western blot with β-actin, a protein expressed in equal amounts in all cells and used as a control for total protein levels, confirms that the small variation in Stat levels are not associated with variations in the total protein incubated with the antibody (Fig. 2, *B*). These results imply that heightened IL-4–induced IgE production is not related to increased systemic expression of Stat6.

Stat6 activation

The essential role of Stat6 activation in IL-4 signaling and IgE production in vivo has been demonstrated in murine models.[21-23] We hypothesized that some individuals with increased sera IgE may demonstrate evidence of increased systemic Stat6 activation in the setting of elevated sera IL-4 and IgE levels. To examine for Stat6 activation, EMSA was used, and the induction of DNA binding activity was assessed by probing with the GAS element from the IRF-1 gene. As positive controls we

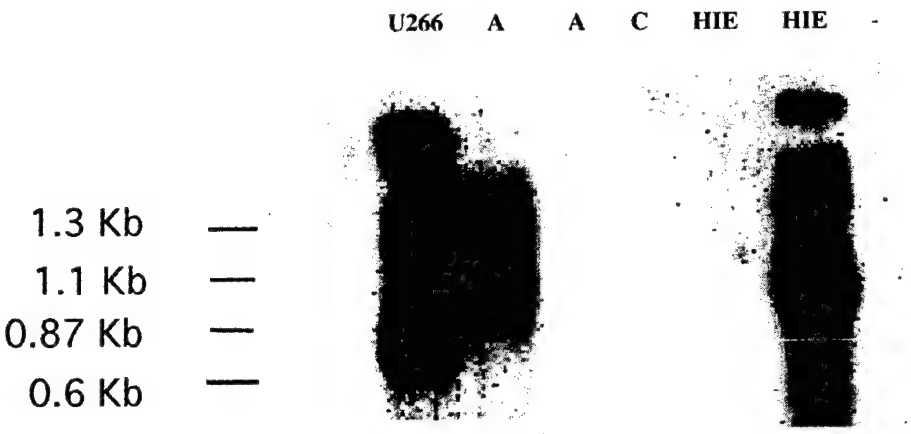

FIG 4. Southern blot of DNA extracted from PBMCs isolated from 2 patients with asthma *(A)*, 2 patients with HIE, and 1 nonatopic control subject *(C)*. U266 (IgE-secreting myeloma cells) served as positive controls. DNA was amplified by using nested primer PCR of switch fragments (Sμ/Sε), and specific products were determined by Southern transfer, hybridization, and incubation with a probe against Sε. Switch fragments are detected in U266 cells, as well as in 2 of 8 patients with asthma (1 case shown in second lane) and 2 of 3 patients with HIE (1 case shown in sixth lane) but not in any of the nonatopic control subjects. The last lane represents no DNA (negative control).

used both Ramos cells and PBMCs isolated from a nonatopic control subject and treated half of these cells with recombinant hIL-4 in vitro. Stat6 was not detected on EMSA in the patient with HIE and the highest measured IL-4 and IgE level (Fig. 3), the other 2 tested patients with HIE, any tested patients with extrinsic asthma and moderately elevated IL-4 and IgE levels, or any of the nonatopic control subjects. These results demonstrate that despite the presence of elevated circulating IL-4 levels, subjects with elevated IgE do not exhibit evidence of systemic Stat6 activation. We also assayed for the presence of germline-ε transcripts as another marker for cells undergoing active class switching to Cε after Stat6 activation[16,50] and found these were absent in all patients and nonatopic control subjects tested (data not shown). These data imply that in these 2 IgE-mediated diseases, either the peripheral B cells have already isotype-switched to IgE or that intracellular signaling is not induced systemically by the increased IL-4 in the circulating environment.

Switch recombination

The absence of detectable systemic Stat6 activation and production of germline-ε transcripts despite elevated sera IL-4 and IgE levels may imply that B cells in the blood of patients have already undergone class switching. If this were the case, switch junction fragments (Sμ/Sε) representing DNA rearrangements and deletional recombination would be present. Such fragments have been found in human B cells in the presence of IL-4 infected with the Epstein-Barr Virus[51] or stimulated with anti-CD40 antibodies.[35] To assess for the presence of switch-junction fragments, DNA was derived from the IgE-secreting myeloma cell line U266 and used as a positive control, and it was extracted from the PBMCs of the patients and nonatopic control subjects. Nested PCR and Southern blot analysis of all specimens was performed with primers derived from Sμ/Sε fragments and incubated with a P^{32}-labeled probe to Sε. Switch fragments are amplified from DNA isolated from PBMCs derived from 2 of 8 patients with asthma, 2 of 3 patients with HIE, and 0 of 14 nonatopic control subjects (Fig. 4). Switch fragments are not amplified in the patient with HIE and the greatest measured IL-4 and IgE level. Therefore cells are present in the blood of these patients that have undergone class switching to IgE.

DISCUSSION

The characterization of cytokine signaling and the JAK-STAT pathway that are critical for IgE class switching in mice has furthered our understanding of the mechanisms of IgE regulation. Our aim was to study mechanisms of cytokine-mediated intracellular signal transduction and IgE class switching in human diseases characterized by elevated levels of IgE. We hypothesized that evidence of systemic cytokine-mediated intracellular signal transduction and IgE class switching could be detected in some individuals with extrinsic asthma and HIE. In this study sera IL-4 levels are significantly elevated in subjects with HIE. The increased level of IL-4 measured in the sera in these patients contrasts with other studies that have examined IL-4 production from CD4+ T-cell clones derived from patients with HIES[41] or assessed IL-4 production after in vitro stimulation with mitogens.[41,42] The data imply that the cellular source of IL-4 is unclear. One possibility is that in vivo stimuli exist that induce secretion of this cytokine. An alternative possibility is that the source of IL-4 in these patients are either T cells present in other organs or other cells, such as basophils or mast cells. A third interpretation is that IL-4 production differs between HIES and HIE.

IL-13, another T_{H2} cytokine, has been shown to induce IgE class switching in vitro,[5-7] but it has not been well studied during human IgE-mediated disease. In both asthma and HIE, the systemic expression is not increased, suggesting that IL-13 plays no significant role in inducing heightened IgE production in these diseases. These results also suggest that therapy for HIES or its variants could be targeted towards inhibiting the T_{H2} cytokine IL-4 alone. Furthermore, even though one may predict that increased IL-4 and IgE levels are related to downregulated IFN-γ levels, no consistent pattern of IFN-γ expression is detected among both groups of patients. This finding challenges the model that T_{H1}- and T_{H2}-type cytokines regulate class switching to IgE in vivo by antagonistically regulating transcriptional activity at the germline-ε promoter. However, it is compatible with the mixed cytokine data reported in previous studies on patients with asthma,[37-39,45,46] suggesting either that these cytokines do not necessarily act in a strictly polarized manner in vivo or that variations may be attributed to other factors such as severity or chronicity of disease. Also, the absence of decreased IFN-γ in HIE provides new data on cytokine expression in HIE and indicates that downregulation of T_{H1} cytokines and IFN-γ signaling is not prominent in these cases.

In addition to examining cytokine expression during IgE-mediated diseases, our aim was to conduct the first study of intracellular cytokine signaling and the role of STAT transcription factors in IgE class switching in humans. The genetics of HIES and its variants are not well known, and therefore we hypothesized that some individuals with markedly increased IgE levels would exhibit increased Stat6 protein expression. The presence of unaltered protein levels here implies that this is not the important genetic defect in HIE or HIES or that this population is heterogeneous.

Another question addressed in this study is whether T_{H2} cytokines in the circulating blood are able to activate Stat6 systemically in IgE-mediated disease. These data demonstrate that despite the presence of increased systemic expression of IL-4 and IgE, Stat6 is not activated in PBMCs. Because IL-4 alone can induce Stat6 activation and the production of germline-ε transcripts in human B cells in vitro,[52] it is unclear why Stat6 activation was not detected in the patients examined. One possibility is that synergistic costimulatory signals provided by CD4+ T cells, such as CD40 ligand, are required for the induction of Stat6 activation to detectable levels.[25] A second explanation is that a subset of B cells present in the blood are induced by IL-4, but our assays are not sensitive enough to detect this activity.

Because increased sera IgE levels do not appear to be the result of active class switching in the blood, one could postulate that other sources or mechanisms may be involved. One explanation is that IgE is being produced by cells that have already undergone class switching (ie, "memory" B cells). The detection of switch junction fragments in several subjects, a requisite precursor to IgE heavy-chain polypeptide production, implies that cells are present in the blood that have already class switched to IgE.

An additional explanation for the absence of active class switching to IgE in the blood is that the cellular source may be the spleen or lymph nodes in the case of HIE or the airways where antigen sensitization occurs in the case of asthma. Indeed, it is clear that T_{H2} cytokines are elevated in the bronchoalveolar lavage fluid or transbronchial biopsy specimens of patients with allergic asthma[53-57] and in the nasal mucosa of patients with symptomatic allergic rhinitis.[43,58,59] Furthermore, germinal centers within the parenchyma of inflamed and hyperresponsive lungs after airway antigenic challenge have been identified in mice (ie, bronchus-associated lymphoid tissue) and shown to produce IgE.[60] Only a few studies have compared the systemic versus local T_{H2} immune response either temporally or quantitatively. In a murine model of allergen-induced asthma, IL-4 levels after antigenic challenge peaked in bronchoalveolar lavage fluid later, although to a similar level to that of the sera.[61] After experimental allergen bronchoprovocation, another group demonstrated that peripheral blood T_H cells decreased in association with their selective increase in the lung.[62] A plausible corollary is that the expression of T_{H2}-type cytokines in the airways during the inflammatory response of late-phase asthma or allergic rhinitis may be later or greater than the systemic expression. This pattern has been described for IL-13.[43] Therefore it is likely that the activation of the JAK-STAT pathway is occurring locally in patients with allergic airway disease but not systemically.

In summary, even though increased IgE production correlates with elevated sera IL-4 levels, it is not associated with increases in the protein expression of Stat6 or the prevalence of Stat6 activation. These results suggest that active intracellular cytokine signaling is not occurring systemically in either extrinsic asthma or HIE. In comparison, the presence of switch fragments in several patients suggests that PBMCs in some individuals may have already class switched to IgE, even if the process is not active. These results imply that increased IgE antibodies may reflect B cells that have already undergone class switching or that cytokine-mediated Stat activation and IgE induction may occur predominantly in target organs in IgE-mediated disease.

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Immune Modulating Agents

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Molecular Mechanisms Controlling Immunoglobulin E Responses

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I. INTRODUCTION

Immunoglobulin E (IgE)-mediated allergic reactions comprise the classic type I immediate hypersensitivity reactions that occur within seconds to minutes of exposure to allergens or antigens. These immune responses can result in systemic anaphylaxis, wheal and flare reactions, allergic rhinitis, food allergies, or bronchial asthma. The underlying mechanism that links these reactions is the binding of IgE to high-affinity Fc receptor (FcεRI) on the surface of mast cells or basophils and cross-linking of FcεRI/IgE complex by antigens. These events trigger the release of several different inflammatory mediators such as vasoactive amines, chemotactic factors, and cytokines that in turn are responsible for the varied symptoms of allergic disease. The route of allergen exposure (e.g., inhaled, intravenous, ingested), the dose, and individual genetic predispositions all can modulate the type or site of reaction that an individual may experience.

The ability of B lymphocytes to produce IgE in response to antigens relies on a complex set of molecular events, several of which have been delineated only recently. One key advance to the understanding of this pathway was the identification of distinct phenotypic subsets of $CD4^+$ T helper cells, termed Th1 and Th2 cells. It appears that Th2-"type" T cells secrete cytokines that are responsible for stimulating an IgE response. Another advance was learning how the cytokines these cells secrete are able to initiate and regulate signals required for B cells to undergo immunoglobulin (Ig) heavy-chain class switching to IgE. In this chapter we will review the molecular pathway that leads to IgE synthesis, beginning with Th2-secreted cytokines that bind to their cell surface receptors on resting B cells and trigger tyrosine phosphorylation of the receptor complex, activating intracellular signal transducing proteins. These proteins in turn stimulate specific transcriptional events that lead to Ig heavy-chain class switch recombination, resulting in an Ig heavy-chain locus that can produce messenger ribonucleic acid (mRNA) encoding IgE heavy-chains and ultimately allergic immune response.

II. T HELPER SUBSETS AND CYTOKINES

In 1986, Mosmann, Coffman, and colleagues [1] reported that murine $CD4^+$ T cell clone lines can be classified into subsets defined by unique patterns of cytokine synthesis and secretion. Type 1 T helper (Th1) cells secrete interleukin-2 (IL-2), tumor necrosis factor (TNF)-β, and interferon-gamma (IFN-γ), whereas type 2 T helper (Th2) cells can secrete IL-4, IL-5, IL-10, and IL-13. Both subsets are capable of producing IL-3, IL-6, granulocyte monocyte colony-stimulating factor (GM-CSF), and TNF-α. A third subset, referred to as ThO cells, exhibit combined type 1 and type 2 patterns of cytokine secretion and comprises the predominant subset isolated from human peripheral blood lymphocytes [2,3]. Type 1 cytokines are important for delayed-type hypersensitivity immune responses, contact sensitivity, macrophage activation, and heavy-chain Ig class switch to IgG2a and IgG3. In contrast, type 2 secreted cytokines provide essential helper function for humoral immune responses and can induce Ig class switch to IgG1 and IgE [4] (Figure 1).

Both Th1 and Th2 cells are believed to arise from a common progenitor T cell population that only secretes IL-2 on antigen stimulation. These naive progenitor Th cells are termed Thp. The generation of a polarized Th1 or Th2 response from naive Thp cells is influenced by the nature of the cytokines present at the time of initial antigen stimulation. For instance, several groups have shown that the presence of IL-4 at the time of antigen stimulation increases the frequency of IL-4-(Th2)-producing cells generated [reviewed in Refs. 5–7]. The presence of IL-4 also inhibits the development of IFN-γ-producing Th1 cells [8]. In addition to IL-4, another Th2 cytokine, IL-10, can decrease the production of IFN-γ in antigen-stimulated Th culture systems. In contrast to IL-4, which appears to mediate its effects on Th development by directly influencing T cell differentiation, IL-10 appears to decrease priming for IFN-γ production by its activity on antigen presenting cells [9].

Several studies have demonstrated that the cytokines IL-12 and IFN-γ increase the production of Th1 cytokines and diminish the production of Th2 cytokines (reviewed in Ref. 5). Interleukin-12 has clearly been shown to increase the priming

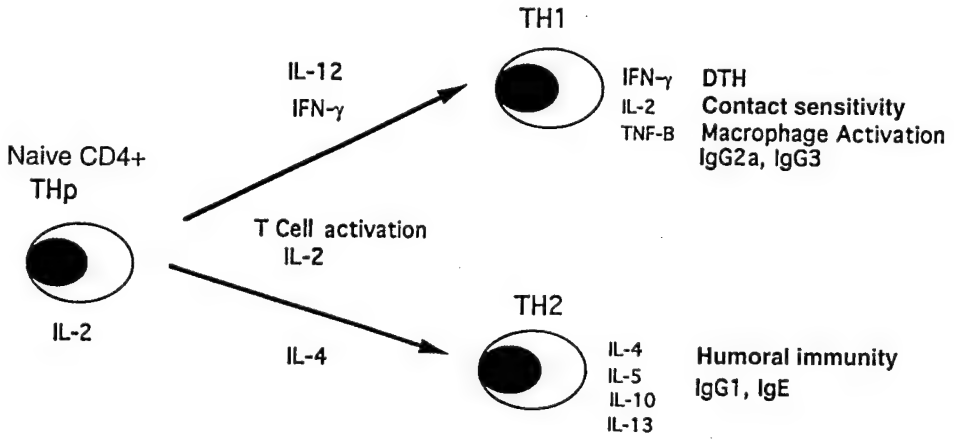

Figure 1 T helper subsets and their cytokines. DTH, delayed-type hypersensitivity.

for IFN-γ (Th1) production at the time of antigen stimulation [10,11]. There is good evidence, both in vitro and in vivo, that IFN-γ can decrease the production of IL-4 during an immune response. Whether this effect is secondary to an inhibition of differentiation of Thp cells into Th2 cells, or to an inhibition of the growth of Th2 cells, is not clear. In addition, conflicting data exist as to whether IFN-γ by itself can enhance priming for IFN-γ production [6,7,12]. Thus, the role IFN-γ plays in regulating Th differentiation remains less certain.

In addition to the developmental effects, Th cell cytokines can alter the growth and proliferation of differentiated T cells. These effects are thought to be important in the cross-regulation of different Th cell populations. For example, IFN-γ inhibits the proliferation of Th2 cells but has no effect on Th1 cellular proliferation [13,14]. It has been proposed that this selective antiproliferative activity may be the mechanism that allows IFN-γ present at the time of antigen stimulation to inhibit the outgrowth of any Th2 cells that were generated in response to antigen stimulation and allows cells that produce IFN-γ (which include Th1 cells, natural killer [NK] cells, $CD8^+$ subsets) to suppress the development of a Th2 response. Alternately, IFN-γ producing cells may inhibit an ongoing secondary Th2 response through the antiproliferative effects of IFN-γ. A second example of the selective effects of cytokines on Th proliferation is the finding that IL-4 can support the growth of Th2, but not Th1, cells [3,15]. This relationship raises the possibility that the defect in Th2 responses reported in IL-4 knockout mice [16,17] may, in part, be due to the requirement of this cytokine for Th2 cellular proliferation. In effect, through self-amplification and cross-regulation, Th subsets can modify and skew an immune response toward a specific and polarized pathway. Finally, costimulatory signals have been shown to help regulate Th differentiation both in vitro [18] and in vivo [19]. For example, in experimental allergic encephalomyelitis (EAE), anti-B7-1 antibodies decreased the incidence of disease in the setting of increased IL-4 production, while anti-B7-2 antibodies exacerbated the disease severity [20]. Anti-B7-2 antibody treatment also suppressed the disease progression in the nonobese diabetic (NOD) model of autoimmune diabetes [21], supporting a role for B7-2 costimulation in the initiation of autoimmune disease.

One important example of the relevance of the polarized Th response occurs in the regulation of immunoglobulin isotypes. The IFN-γ produced by Th1 cells promotes the selective production of IgG2a and IgG3, the principal antibodies involved in opsonization and phagocytosis of microbes, and suppresses the production of IgE and IgG1. In contrast, type 2 cytokines promote the selective production of IgG1 [22,23], which contributes to the neutralization of toxins produced by extracellular bacteria, and of IgE, which is critical to mast cell degranulation (reviewed in Ref. 4). Hence, the ability of the immune system to produce isotype-specific humoral antibodies relies on the differential production of cytokines and is a key factor in the development of diverse biological responses.

III. TH2 CYTOKINES IN ALLERGIC DISEASE

The Th type 2 cytokines IL-4 and IL-13 are the key mediators in Ig class switching to IgE and therefore are important for IgE-dependent mast cell degranulation. Much of the early evidence for this was derived from finding that the presence of

IL-4 was necessary for class switching to IgE by B lymphocytes, observed in several in vitro [22,23] and in vivo (reviewed in Ref. 24) models. In addition, mice homozygous for a mutation in the IL-4 gene were unable to produce substantial IgE [16,17]. More recently, it has been demonstrated that a second cytokine produced by human Th cells can stimulate IgE production. This cytokine, IL-13, shares both considerable sequence homology and biological activity with IL-4, including IgE synthesis [25,27]. Although the human IL-13 shares significant homology with the murine equivalent [28], the murine protein is not important for IgE production, likely because of the lack of response of murine B cells to IL-13.

Further, considerable evidence has accumulated supporting the model that the pathogenesis of IgE-mediated allergic diseases, such as rhinitis and extrinsic asthma, involves heightened Th2 mediated immune responses. For example, the great majority of allergen-specific $CD4^+$ T cell clones derived from peripheral blood lymphocytes of patients with allergies express a Th2 and ThO phenotype consisting of high levels of IL-4 and IL-5, and little or no production of IFN-γ [29,30]. Interleukin-4 and IL-5, but not IFN-γ, levels are elevated in sera of patients with allergic asthma [31,32]. Also, allergic asthmatics' peripheral blood possesses an increased number of B cells bearing the IgE receptor CD23, which is evidence of B cell activation and IL-4 and IL-13 up-regulation [33].

The presence of activated Th2 cells in target organs during human allergic disease has been well documented. As examples, increased numbers of activated Th2 lymphocytes and mast cells expressing IL 4 mRNA or producing IL-4 have been assayed from bronchoalveolar fluid [34,35] and nasal mucosa [36,37] of patients with symptomatic allergic asthma and rhinitis, respectively. These results can be replicated experimentally after allergen inhalation challenge, but not after diluent challenge in atopic patients [38]. Further, these results are specific to allergic disease, as exemplified by one report which measured increased levels of IL-4 and IL-5 in the bronchoalveolar lavage fluid of patients with allergic asthma, in contrast to increased IL-2 and IL-5 measured in bronchoalveolar lavage fluid of patients with nonallergic or intrinsic asthma [39]. Interestingly, a close correlation between the number of activated T cells and severity of atopic asthma has been demonstrated in both blood and bronchial fluid [40).

More recently, IL-13 has been detected in target organs during allergic disease. Although it has been difficult to measure IL-13 in the peripheral blood during allergic exacerbations [41], IL-13 mRNA and IL-13 transcripts have been measured in the bronchoalveolar lavage cells of atopic patients after segmental bronchial allergen challenge [42] and in the nasal mucosa of patients with perennial allergic rhinitis [41]. The role of IL-13 expression in vivo is not yet well defined. This expression of IL-13 predominantly in the target tissues may be a manifestation of a more limited local role of 1L-13 in IgE regulation rather than a systemic one. But in some circumstances, the presence of IL-13 may substitute fcr IL-4 in promoting IgE synthesis. In support of this latter model is one study which described that "naive" ($CD4^+45RO^-$) human T cells can be primed in vitro, to secrete large amounts of IL-5, IL-13, and IFN-γ, but not IL 4, and these cells can induce IgE production efficiently. This IgE response was blocked completely by neutralizing anti-IL-13 antibodies, but not by anti-IL-4 [43].

Whereas IL-4 is critical to IgE production, another type 2 cytokine, IL-5, acts as a differentiation and growth factor for eosinophils during allergic disease.

Interleukin-5, IL-3, and GM-CSF all induce eosinophil production, as demonstrated in both in vitro and in vivo experiments [44], but only IL-5 is specific for the eosinophil lineage. The IL-5 stimulates eosinophilopoiesis in the bone marrow [45] and promotes the terminal differentiation of myeloid precursors into eosinophils [46]. Eosinophils play an essential role during allergic diseases and parasitic infections by releasing eosinophil-derived proteins such as major basic protein (MBP) and the eosinophilic cationic protein (ECP). In helminthic and parasitic infections, the eosinophilie-derived granules are believed to be toxic to the infesting cells. In asthma, epithelial shedding is believed to result from the release of eosinophil major basic protein [47], and mucus hypersecretion and airway hypereactivity are believed to be consequences of the release of the eosinophil and mast cell–derived leukotriene C4, which is cleaved to the active products leukotriene D4 and E4 [48]. In addition, IL-5 promotes the migration of eosinophils from the blood to the tissues in response to antigen challenge [49,50] and increases eosinophil, but not neutrophil, adhesion to vascular endothelium [51]. Although IL-5 does not contribute to IgE production, the Th2 immune response that promotes IL4, IL-13, and IL-5 secretion, all contribute to the inflammation-related symptoms in complementary ways.

IV. JAK–STAT PATHWAY

Many of the biological activities regulated by cytokines arise from their ability to induce the transcription of target genes rapidly. Cytokines initiate this regulation by binding to their cell surface receptors and activating intracellular signaling molecules that then can induce transcription. The JAK–STAT pathway is one important signaling pathway responsible for transcription of specific genes in response to cytokines and growth factors. Binding of cytokines to their receptors results in the oligomerization of the receptor proteins. These oligomers can be dimers (homo or hetero), trimers, or tetramers, depending on the cytokine. This oligomerization of the receptors results in the activation, likely by transphosphorylation, of the Janus kinase (JAK) family of tyrosine kinases, which are physically associated with the cytoplasmic tails of the cytokine receptors and exert a low basal level of kinase activity constitutively. To date, the JAK family is known to comprise four kinases, referred to as JAK1, JAK2, JAK3, and Tyk2. They are expressed ubiquitously, except JAK3, which is primarily expressed in hematopoietic cells (reviewed in Ref. 52). The activation of the receptor-associated JAKs then leads to the phosphorylation of tyrosine residues within the cytoplasmic tail of the receptor proteins. These phosphorylated tyrosines in turn serve as docking sites for the binding of specific signaling proteins, the signal transducer and activator of transcription (STAT) proteins, via interactions between the receptor phosphotyrosyl motifs and src homology 2 (SH2) domains of the STATs. The STAT proteins, once recruited to the receptor complexes, become activated via tyrosine phosphorylation by the JAKs present in the receptor complex. Subsequently, the STATs form homo- or heterodimers, dissociate from the receptor complexes, and translocate into the nucleus, where they activate transcription of genes whose promoters contain STAT binding sites. Many of these STAT binding sites were initially characterized as elements important for the activation of gene expression by IFN-γ and were therefore termed gamma acti-

vation site (GAS) elements (reviewed in Refs. 52–54). It is now clear that many of these elements can act as response elements for other cytokines.

The JAK–STAT signaling pathway for the interferons was the first to be studied extensively. The cell surface receptor for IFN-γ is composed of a heterodimer of an α chain, which is associated with JAK1, and a β chain, which is associated with JAK2. Although the presence of only the α component allows a cell to bind IFN-γ it is not sufficient to generate a biological response (as measured either by IFN-γ-stimulated transcription or antiviral responses) [55,56]. Experiments in which the β subunit was transfected into cells that only expressed the α subunit demonstrated that the β subunit was necessary for these cells to activate the JAK–STAT signaling pathway and generate antiviral responses [55,56]. This β subunit, also referred to as accessory factor-1 (AF-1), is absent in Th1 cells, but not Th2 cells, and is believed to be the reason underlying the inability of Th1 cells to activate the JAK–STAT signaling pathway in response to IFN-γ [57]. After binding to its cell surface receptor, IFN-γ stimulates the activation of JAK1 and JAK2, leading to the phosphorylation of the receptor proteins. The phosphorylated tyrosines of the α chain act as a docking site for the binding of Stat 1. Activated Stat 1 then homodimerizes and translocates into the nucleus, where it acts as a transcriptional activator for several IFN-inducible genes [54,58,59].

It is now recognized that other cytokines share this general model of signal transduction, and that at least seven members of the STAT family exist (Stats 1, 2, 3, 4, 5a, 5b, and 6). These proteins share significant homology over several functional domains. The greatest homology is observed within the regions of the SH2 and SH3 motifs and the deoxyribonucleic acid (DNA) binding domain (reviewed in Ref. 60) (Figure 2). Nonetheless, different cytokines/ligands are able to activate different STATs with great specificity. Specificity of signaling is achieved at the level of the cytokine receptor/STAT interaction and not regulated by the particular JAKs involved. This specificity results from the interaction between the SH2 domain of the STAT and motifs on the receptor chains that, after activation by ligand, contain phosphorylated tyrosine residues [61]. This interaction allows the recruitment of different STATs into different receptor complexes.

The signal transduction pathway activated by IL-4 and IL-13 both utilize the Stat 6 molecule. Originally Stat 6 was purified and cloned from nuclear extracts of IL-4-stimulated human myeloid cells. These nuclear extracts contained DNA binding activity capable of specific interaction with a GAS element. When this activity was purified, it was found to be a specific 100 kD polypeptide that functions in a dimeric state and reacts with an antibody that recognizes phosphotyrosine, confirming its requirement for tyrosine phosphorylation [62]. In hematopoeitic cells, IL-4

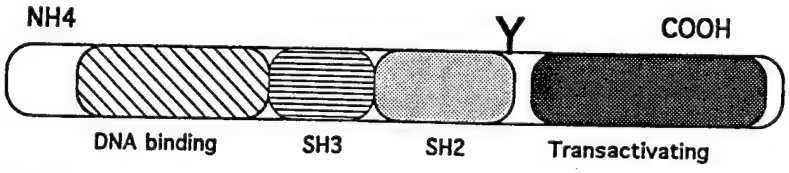

Figure 2 Signal transducer and activator of transcription (STAT) protein. Y, tyrosine residue.

binds to a cell surface receptor complex that consists of a heterodimer of the ligand-specific α chain (termed IL-4Rα) and the common γ (γC) chain that is also a component of the receptors for IL-2, IL-7, IL-9, and IL-15. Dimerization leads to the activation of JAK1, which is associated with the IL-4Rα, and JAK3, which is bound to γC. The activation of these JAKs results in phosphorylation of several tyrosine residues contained within the cytoplasmic tail of the IL-4Rα. Then Stat 6 can be drawn into the receptor complex by interaction between the SH2 domain of Stat 6 and one of several phosphotyrosyl motifs of the IL4Rα. Stat 6 becomes tyrosine-phosphorylated, allowing it to form homodimers using two reciprocal SH2-phosphotyrosyl interactions. Homodimers of Stat 6 can then translocate into the nucleus, where they bind to two distinct types of elements. The Stat 6 can bind to, but not activate transcription from GAS elements that contain the sequence TTC N(2–3) GAA. In addition, Stat 6 can bind to the sequence TTC N(4) GAA [63,64]. This sequence, which does not bind other STATs, is found in the promoter of IL-4-inducible genes, such as the CD23 gene and the Ig heavy-chain germline epsilon (ϵ) gene (discussed later) [65,66] (Figure 3). Proof of the importance of Stat 6 to IL-4-mediated signal transduction came from mice which lack expression of Stat 6 as a result of gene targeting of the Stat 6 gene. Mice deficient in Stat 6 by gene targeting are unable to activate several immune events normally induced by IL-4 such as the activation of a Th2 proliferative response induction of CD23 and MHC class II on B cells, nor can they produce significant IgE and IgG1 responses after helminth infection [67,69].

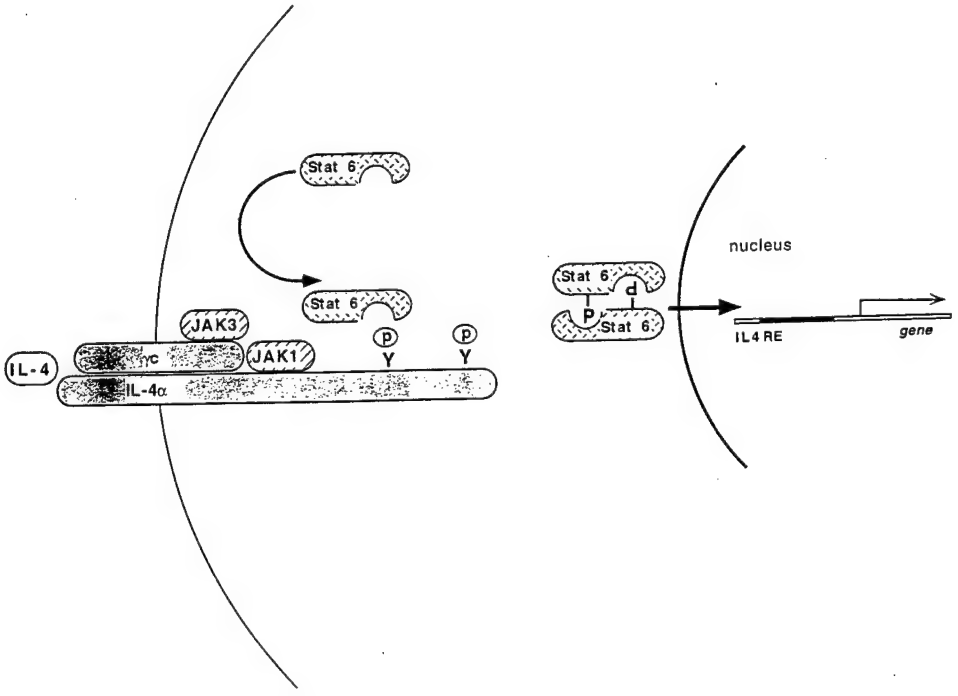

Figure 3 IL-4-mediated Stat 6 signal transduction pathway. IL4RE, interleukin-4 response element; PY, phosphorylated tyrosine.

It was believed that the IL-13 receptor was composed of the α chain that is common to the IL-4 receptor (IL-4Rα) and associated with JAK1, and a novel component that is not completely defined but differs from the γC chain and is not associated with JAK3 (reviewed in Refs. 70–71). It has since been learned that a lower-affinity receptor component, termed IL-13 receptor α, also is capable of binding to IL-13 but not IL-4 [72]. It also has been proposed that coexpression of the IL-13 α receptor subunit and IL-4Rα can act as a high-affinity receptor complex [72]. Because the IL-4Rα chain is a component of the IL-13 receptor, the downstream signaling initiated by IL-13, including the activation of Stat 6, overlaps with the signaling activated by IL-4.

V. IMMUNOGLOBULIN HEAVY-CHAIN CLASS SWITCHING

Immunoglobulin heavy-chain class switching is the process whereby an activated B cell changes the heavy-chain constant region at the carboxy terminus (CH) of the antibody it produces to alter its effector function while still retaining its antigenic specificity. In general, Ig class switching occurs by a deletional recombination event between regions of repetitive DNA, called switch (S) regions, that are present upstream of each heavy-chain constant region, except Cδ. This recombination juxtaposes the fully assembled variable region (composed of rearranged VDJ gene segments) directly upstream of different CH genes that encode for different Ig isotypes. For example, in the case of Ig class switching from IgM to IgE, the Cμ locus and downstream genes are deleted so that the Cϵ gene becomes juxtaposed directly 3′ to the VDJ region and VDJ-Cϵ transcripts are encoded [24].

High levels of Ig class switching require two types of signals. One is a B cell activation signal that follows contact between the B cell surface molecule CD40 and its ligand CD40L expressed by activated Th cells [73]. Recognition of the antigen-specific MHC class II complex by the T cell receptor leads to the expression of CD40L on the Th cells. This interaction occurs in germinal centers of secondary lymphoid tissues and in part prevents the B cells from undergoing apoptosis [74]. Of note is that the X-linked hyperimmunoglobulin M syndrome results from mutations in the genes encoding for CD40L and is characterized by impaired CD40/CD40L interactions, defective class switching, and absence of all immunoglobulin isotype classes except IgM [75].

The second type of signal responsible for Ig class switching involves cytokine-induced expression of particular germline CH transcripts via cytokine activation of the corresponding germline promoter. Immunoglobulin class switching has been shown to be preceded by the expression of germline transcripts in the area surrounding the switch region. Also, certain cytokines have been shown to regulate specific Ig class switching differentially through the induction of particular germline transcripts. For example, it has been demonstrated that the addition of IL-4 to lipopolysaccharide (LPS) cultures of B cells induces high levels of germline ϵ and γ1 transcripts that correlate with the amount of IgE and IgG1 induction, respectively [76,78]. More recently, IL-13 has been shown to induce germline IgE heavy-chain gene transcription in purified B cells, even in the presence of neutralizing anti-IL-4

antibody, confirming its independent role in IgE production [25]. In contrast, IFN-γ has been shown to suppress the IL-4-induced germline ε transcription. Furthermore, regulation of IgE switching seems to occur through these cytokines' antagonistic effects on the germline ε promoter [79]. These observations seem to support a model in which specific heavy-chain germline transcription always precedes switch recombination and in which type 1 versus type 2 cytokines differentially regulate the switching process to Cε.

Although the association between heavy-chain germline transcription and switch recombination is highly correlative, direct proof of a causal relationship has, until recently, been lacking. Germline transcription initiates at an exon (I exon) that is 5′ of the corresponding S region and initially proceeds through the S region and the CH gene. The transcript is processed to a stable form which is relatively conserved among different CH transcripts. This conservation raises the possibility that the transcripts themselves may play an important functional role in switch recombination. Germline transcripts cannot encode large proteins because they contain stop codons in the open reading frame of the CH gene but are believed essential for making the DNA accessible to the recombination machinery (reviewed in Ref. 24). The transcripts may either act as RNA molecules or encode important short polypeptides [80]. An alternative model is that the germline transcripts themselves are not essential but that transcription per se (or perhaps associated topoisomerase activity or chromatin change) is essential for targeting switch recombination to specific heavy-chain loci. A third possibility is that germline transcription is an epiphenomenon of other cytokine-induced events (e.g., switch region recombinases) that are essential for specific switching events.

The importance of germline transcription to class switching has been addressed by gene targeting experiments in which the I region promoters and exons have been removed or replaced. For instance, removal of the Iγ1 element from the heavy-chain locus by homologous recombination markedly inhibited Ig switching to IgG1. Also, heterozygous mice with one wild-type allele and one mutated allele switched to γ1 on the wild-type allele but not the mutated one [81]. In another study, mice lacking the Iγ2b germline promoter could not undergo switching to the IgG2b isotype [82]. These studies suggest that sequences within the Iγ1 or Ig2b locus, including genes encoding promoter activity, are required for switching to IgG1 and IgG2b, respectively. In comparison, mutated B lymphoid cells in which the Ig heavy-chain ε (Iε) locus was replaced by the Ig heavy-chain Eμ enhancer and Vh promoter to allow constitutive transcription through the ε switch region still transcribed the ε locus independently of the presence of IL-4. These cells can switch to ε in the absence of IL-4, but at levels 10-fold lower than that of nonmutated cells cultured with IL-4 [83]. These findings have been confirmed in transgenic mice with the same mutation [77]. These studies suggest that optimal efficiency of the switching process requires the presence of an intact Iε region, implicating factors besides the transcription through the Sε region in the regulation of class switching. Similar studies in which the Iγ1 region was replaced by the metallothioneine promoter also illustrate that providing germline transcripts to a heavy-chain locus can supplant the requirement for cytokine in the switching process and supports a model in which cytokine (e.g., IL-4) targets Ig class switching simply by the induction of these germline transcripts [84].

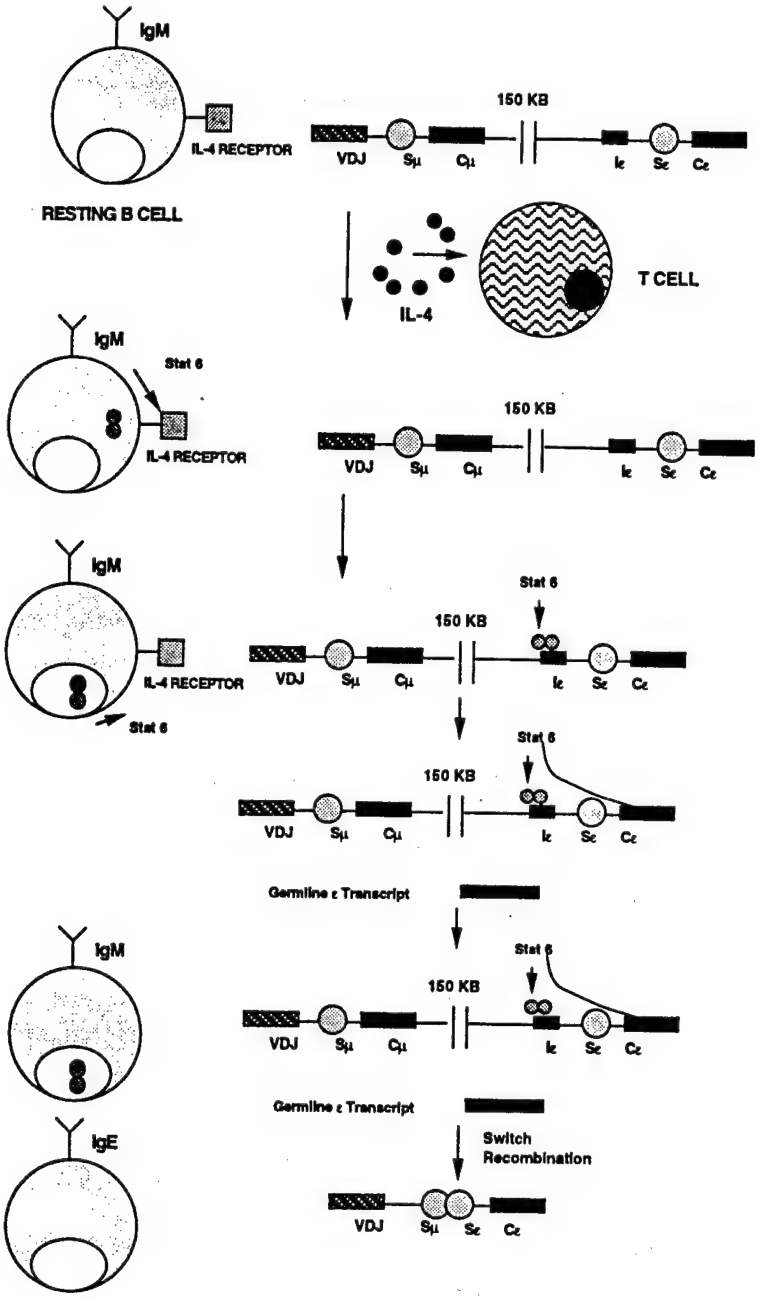

Figure 4 IgE switch recombination. IL-4 binds to its cell surface receptor on the resting IgM^+ B cell. After tyrosine phosphorylation of the receptor complex, Stat 6 is activated, rendering it competent for translocation into the nucleus and binding to specific DNA sequences found in the promoter region, thereby stimulating germline ϵ transcription. A deletional recombination event occurs between S regions, juxtaposing the fully assembled variable region directly beside the $C\epsilon$, and VDJ-$C\epsilon$ transcripts are encoded.

VI. CONCLUSIONS

In summary, IgE production is the end result of highly coordinated and specific immune responses beginning with exposure to allergens in a genetically predisposed individual. Activated Th2 cells secrete IL-4 and IL-13 that bind to their cell surface receptor on the resting IgM^+ B cells. After tyrosine phosphorylation of the receptor complex, Stat 6 is activated, rendering it competent for translocation into the nucleus and binding to specific DNA sequences found in the promoter region, thereby stimulating germline ϵ transcription. After T cell–B cell contact involving CD40L and CD40, a deletional recombination event occurs, resulting in mRNA that encodes for the IgE heavy-chain polypeptide (Figure 4). Immunoglobulin E binds to FcϵRI on mast cells and basophils, allowing cross-linking of FCϵRI/IgE complex by antigens and release of a variety of inflammatory mediators that cause allergic symptoms. What remains unclear at this point is precisely how this pathway is modified during human allergic disease or after exposure to certain antigens and what dictates an individual's genetic predisposition to a heightened IgE production. The 5q31-q33 chromosome locus containing genes encoding for IL-4, IL-5, IL-13 has been associated with clinical atopy, bronchial hyperresponsiveness, and elevated IgE levels [85,87], but delineating how interactions between environmental and genetic influences modify complex immune responses remains a great challenge.

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Identification of a STAT6 Domain Required for IL-4-Induced Activation of Transcription[1]

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Tyrosine phosphorylation of STAT6 in response to IL-4 results in the formation of STAT6 homodimers that bind specific DNA elements. Although binding sites for STAT6 have been shown to be important for the function of several IL-4-inducible promoters, the role of STAT6 in this activation has not been defined. To determine whether STAT6 is a transcriptional activator, different portions of the carboxyl terminus of STAT6 were fused to the yeast Gal4 protein DNA binding domain. Analysis of these chimeric Gal4-STAT6 proteins demonstrates that a 140-amino-acid proline-rich region of the carboxyl terminus of STAT6 contains a region that activates transcription. Truncation mutants of STAT6 that lack this domain cannot activate transcription and are capable of repressing transcription stimulated by a wild-type STAT6 protein. Strikingly, the ability of IL-4 to induce transcription from the Ig germline ϵ promoter is suppressed by overexpression of a carboxyl-terminal deletion mutant of STAT6. These studies demonstrate that the carboxyl terminus of STAT6 contains an activating domain required for the induction of genes by IL-4. *The Journal of Immunology,* **1997, 159: 1255–1264.**

Although IL-4 was identified initially as a B cell stimulatory factor, it has subsequently been shown to activate multiple biologic activities in both hemopoietic and nonhemopoietic cells, and is one of the key cytokines for controlling the host response to antigenic stimulation. For example, IL-4 is a potent regulator of allergic immune responses, both because it increases the number of Th2 cells (reviewed in Ref. 1) and induces B cells to undergo Ig class switching to IgE (reviewed in Ref. 2).

The pleotrophic functions of IL-4 result from its ability to regulate cellular differentiation, proliferation, and apoptosis via the activation of several distinct signaling pathways. Recent work has elucidated much of the basic biology underlying IL-4 signal transduction. Signaling is initiated when IL-4 binds its cell surface receptor that, in hemopoietic cells, consists of a heterodimer of the ligand-specific α-chain (termed IL-4Rα) and the common γ chain (which is also involved in the receptor complexes used by IL-2, IL-7, IL-9, and IL-15) (3, 4). This dimerization leads to the activation of the receptor-associated JAK tyrosine kinases (reviewed in Ref. 5). The activation of the Jak1 (bound to the IL-4Rα) and Jak3 (bound to common γ) tyrosine kinases is associated with the phosphorylation of tyrosine residues within the cytoplasmic tail of the receptor (reviewed in Ref. 6). This modification of the receptor stimulates the binding of different signaling molecules via interactions between these receptor phosphotyrosyl motifs and either phosphotyrosine-binding or *src* homology 2 (SH2)[3] domains of the signaling molecules. These signaling molecules are then activated by tyrosine phosphorylation by one of the JAKs present in the receptor complex. Using IL-4Rα mutants, it has been shown that the activation of different signaling pathways by IL-4 requires different motifs within the cytoplasmic region of the receptor (7–9). The activation of either insulin receptor substrate (IRS)-1 or IRS-2 by IL-4 is essential for IL-4-induced cellular proliferation and depends on the insulin/IL-4R motif (8). Recently, the ability of IL-4 to prevent apoptosis has been shown to involve both IRS-dependent and IRS-independent mechanism (10). In addition to these pathways, the ability of IL-4 to affect cellular differentiation appears to require a different signaling pathway that can regulate the transcription of distinct genes in target cells. This pathway requires the activation of the signaling protein STAT6.

STAT6 is a member of the STAT family of signaling molecules (11–15). The STAT family of signaling molecules now includes seven different family members (STAT1, -2, -3, -4, -5a, -5b, and -6). These factors are activated in response to different cytokines and appear to be essential mediators of cytokine function (reviewed in Refs. 16 and 17). Different STAT molecules share significant homology over various domains that appear essential for their activity. The greatest homology is found in the regions of an SH2 and a putative SH3 motif (reviewed in Ref. 17). There is a conserved tyrosine motif present directly carboxyl to the SH2 domain that is phosphorylated in response to ligand and is essential for STAT dimerization. In addition, there is a DNA-binding motif and a conserved region in the amino terminus of the STATs that has recently been implicated in STAT tetramerization (18).

STAT6 is expressed constitutively in the cytoplasm of most cells and is recruited into the IL-4R complex by interaction of the STAT6 SH2 domain and one of several phosphorylated tyrosine motifs on the cytoplasmic domain of the IL-4R α-chain. Once drawn into the receptor complex, STAT6 is phosphorylated on tyrosine 641, which allows STAT6 molecules to homodimerize

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[3] Abbreviations used in this paper: SH2, *src* homology 2; aa, amino acid; EMSA, electrophoretic mobility shift assay; gal, galactosidase; GAS, interferon-γ-activating site; HuSTAT, human signal transducer and activator of transcription; IRF, interferon-response factor; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; TK, thymidine kinase.

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(19). STAT6 homodimers are capable of translocating to the nucleus, where they can bind to specific DNA elements. STAT6 binding sites have been identified in the promoter regions of several IL-4-inducible genes, including CD23, MHC class II, and Ig germline ϵ and $\gamma 1$ promoters (13, 20, 21). Several of these STAT6 binding sites have been shown to be important elements in the function of these promoters. For example, there are two STAT6 binding sites within the Ig germline ϵ promoter, one of which is essential for the activation of this promoter by IL-4 (22, 23). The germline ϵ transcripts activated by this promoter are essential for Ig class switching to IgE (reviewed in Ref. 2). The importance of STAT6 in mediating IL-4-specific functions has been demonstrated in STAT6-deficient mice, which cannot induce CD23, MHC class II expression, or IgE in response to IL-4 (24–26).

Although STAT6 is clearly important for some of the biologic activity of IL-4, the mechanism by which STAT6 mediates IL-4 effects on target tissues has not yet been defined. STAT6 was characterized initially as an IL-4-inducible DNA-binding complex that bound to either IFN-γ-activating sites (GAS) or IL-4 response elements (11–14, 27). Although homodimers of STAT6 can bind DNA, these molecules have not been shown to be able to activate transcription. Interestingly, when minimal STAT6 binding sites are multimerized and cloned upstream of a minimal TK promoter in a reporter construct, IL-4 does not confer enhancement of transcription to this minimal promoter (23, 28). There are several differences between STAT6 and other STATs in terms of its DNA-binding properties and the ability to activate transcription. First, although the binding sites for STAT1, 3, 4, 5, and 6 conform to the general structure TT(N)nAA, different STATs prefer different spacing between TT and AA. A core palindromic TT-AA motif with a 5-bp spacing can bind all of the STATs (28, 19). In contrast, the STAT6 complex is the only STAT complex that can bind a 6-bp-spacing TT-AA structure (28). Virtually all of the functional IL-4-responsive elements identified conform to this $TT(N)_6AA$ consensus. Second, although several other STATs have been shown to contain *trans*-activating domains in their carboxyl terminus (29, 30), this region of STAT6 shows no homology to the carboxyl terminus of other STATs. These differences have led us to examine the function of the carboxyl terminus of STAT6. The studies described below identify a transcriptional activating domain in the carboxyl terminus of STAT6 and demonstrate that this domain is required for the induction of genes by IL-4.

Materials and Methods

Cells, cytokine, and Abs

The 293 cell line (human embryonic kidney cells) is a gift of Dr. Chris Schindler, Columbia University (New York, NY), and was grown in DMEM plus 10% FCS. M12.4.1 B cell lymphoma cell line (20) was maintained in RPMI plus 10% FCS. The NIH3T3 cell line is a gift of Dr. Katherine Calame (Columbia University) and is grown in DMEM plus 10% FCS. DND39/L12, which expresses luciferase under the control of the germline ϵ promoter in an IL-4-dependent manner, is derived from the DND39 human Burkitt lymphoma B cell line (31). It was made by cloning a 637-bp fragment of the germline epsilon promoter (bp 8–644 of accession X56797) upstream of the luciferase gene in the plasmid pLuxF49, an ampicillin-resistant PUC-based plasmid containing luciferase, followed by the SV40 poly(A) and splice sequences (D. Lloyd and J. Thompson, Pfizer, Groton, CT, unpublished observation). This plasmid was cotransfected into DND39 with pRSV-Neo by electroporation, and clonal, G418-resistant cell lines were selected, one of which was DND39/L12. Human rIL-4 is a gift of Dr. Satwant Narula at Schering-Plough Corp. (Kenilworth, NJ) and was used at 100 U/ml. The M-20 anti-STAT6 Ab and anti-Gal4 DNA-binding domain Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 11503 is anti-HuSTAT6 polyclonal Ab generated against the SH2 domain of HuSTAT6.

Plasmids and transfection procedures

Plasmids pSV40-Gal4(1-147), $pGal_5$-Luc, and pSV40-Gal4(1–147)-vp16 are gifts of Dr. Katherine Calame. Plasmids in which different portions of murine STAT6 protein carboxyl region fused to the Gal4 DNA binding domain were made by fusing PCR-amplified cDNA fragments in-frame to the carboxyl end of the Gal4 DNA binding domain. Detailed subcloning procedures and plasmid maps are available upon request. Full-length STAT6 was cloned initially by PCR from the published sequence (14) into the baculovirus vector pVL1393 (PharMingen, San Diego, CA) and then fused to a C-terminal *myc* epitope tag (SGRSMEQKLISEEDLN) to create pVL1393-STAT6.*myc*. The expression vectors are pcDNA3 based (Invitrogen Corp., San Diego, CA). The full-length HuSTAT6 cDNA was cut out from pVL1393-STAT6.*myc* by *Eco*RV and *Kpn*I, blunted by Klenow fill-in, and subcloned to the *Eco*RV site on pcDNA3. HuSTAT6-Δ(804–847) expression plasmid was constructed by Klenow blunt and insert the *Eco*RV-*Hin*dIII fragment from pVL1393-STAT6.*myc* to pcDNA3 cut by *Eco*RV. HuSTAT6-Δ(659–847) was constructed by cutting out a *Eco*RV-EclI fragment from pVL1393-STAT6.*myc*, blunted by Klenow fill-in, and subcloned into *Eco*RV-linearized pcDNA-3. $p(I\epsilon\text{-IL4RE})_4$-Luc was constructed by subcloning a tetramerized C/EBP/STAT6 site in front of a TK minimal promoter-driven luciferase. The single C/EBP/STAT6 site is made by annealing two complementary oligonucleotides. The sequences of the two oligomers are 5′-GATCTCTGTTCTTGGGAAGTTGACTAAG GCA-3′ and 5′-GATCTGCCTTAGTCAACTTCCCAAGAACAGA-3′, respectively. pSV40-βgal is a gift of Dr. Chris Schindler. pMEP4-HuSTAT6-Δ(659–847) is constructed by subcloning *Kpn*I-*Xba*I fragment of pcDNA3-HuSTAT6-Δ(659–847) into pMEP4 vector (Invitrogen Corp.) cut by *Kpn*I-*Xba*I.

293 cells and NIH3T3 cells were transfected by standard calcium precipitation protocol (32) with some modifications. NIH3T3 cells were kept in calcium precipitate overnight, then washed and cultured in fresh medium overnight. The cells were harvested 48 h after transfection, and luciferase activity was measured. 293 cells were also kept in the calcium precipitate overnight. The cells were split on the second day, and fresh medium was added with or without human IL-4. The cells were harvested 48 h after transfection, and luciferase activity was measured. Transfection of M12.4.1 was done by electroporation. Briefly, the cells were electroporated at 220 V, 960 μF at 12 million cells/ml in RPMI medium. The cells were harvested after 48 h. DND39/L12 cells were electroporated at 240 V, 960 μF at 12 million cells/ml in RPMI medium. The cells were kept in RPMI for 48 h, and then selected with hygromycin B (Boehringer Mannheim Corp., Indianapolis, IN) at 400 μg/ml plus 50 μM EDTA.

Electrophoretic mobility shift assay (EMSA)

The gel shift assay was described before (12). Briefly, the cells were treated with human IL-4 for 15 min at 37°C, and the whole cell extract was made in lysis buffer, 0.5% Nonidet P-40, 50 mM Tris, pH 8, 200 mM NaCl, 10% glycerol, and 0.1 mM EDTA. Binding was done in 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 20 mM HEPES, pH 7.9, 4% Ficoll, 0.5 mM DTT, 1.2 mg/ml BSA, 160 μg/ml dI:dC, and 5 μg of the extract. The Ab supershift was done by adding 2 μg of M-20 Ab or preimmune serum to the binding reaction. The IRF-1 (interferon response factor 1) GAS probe spans bp 138 to −107 of the IRF-1 promoter TACAACAGCCTGATTT CCCCGAATGACGGC. The complex was resolved in 4.5% PAGE.

Luciferase assay and β-gal assay

Luciferase assay and β-gal assay were described previously (28).

Northern blot analysis

DND39/L12 cells were stably transfected with either the empty vector pMEP-4 or pMEP-4-HuSTAT6-Δ(659–847). The cells were induced by 1 mM of $CdCl_2$ for 24 h and RNA isolated from cells that were either treated with IL-4 or left untreated for 3 days. The probe used to detect germline ϵ transcript was isolated from the pBS-I-4 plasmid and contains exon 1 of the germline ϵ gene and part of Cϵ (33). The DNA probe was labeled with Rediprime random prime labeling kit (Amersham International, Little Chalfont, Buckinghamshire, U.K.).

Results

The carboxyl-terminal proline-rich region of STAT6 contains a trans-activation domain

Although murine and human STAT6 proteins share 83% identity at the amino acid level (15), they differ somewhat in the carboxyl-terminal region. Interestingly, the carboxyl-terminal region of both

human and murine STAT6 are both proline rich (Fig. 1*A*), which is a hallmark for some classes of *trans*-activation domains (34). Recent work has shown that the carboxyl-terminal region of some other STAT proteins (i.e., STAT2 and STAT5) contain *trans*-activating domains (30, 35). We therefore focused our investigation for a STAT6-activating domain on the carboxyl portion of the molecule.

To determine whether STAT6 contains a region capable of activating transcription, we used the modular property of transcription factors to generate fusion proteins in which portions of STAT6 were fused to the DNA-binding domain of a heterologous protein, Gal4 (36). Both human (aa 629–847) and murine (aa 596–837) STAT6 carboxyl-terminal region were fused to the Gal4 DNA binding and dimerization domain (aa 1–147). Expression vectors containing these fusion constructs were cotransfected into NIH3T3 cells along with a reporter plasmid ($pGal_5$-luc) that contains five Gal4 binding sites upstream of the minimal E1B promoter and the luciferase reporter gene. Extracts were harvested 36 h after transfection and assayed for luciferase activity. The expression of all fusion proteins was equivalent, as assayed by Western blotting (using an Ab specific for the Gal4 DNA binding domain) of extracts isolated from these transfectants (data not shown).

The addition of the carboxyl-terminal regions of either the human or murine STAT6 imparts transcriptional activating properties (as measured by luciferase activity) to the Gal4 DNA binding domain in these Gal4-STAT6 fusion proteins (Fig. 1). The level of activation by these Gal4-STAT6 fusion proteins is one-third that of the activity of the Gal4-VP16 fusion protein (Fig. 1*C*). Interestingly, the level of activation by these Gal4-STAT6 fusion proteins is not affected by the addition of IL-4 to the cultures (data not shown). These experiments demonstrate that the carboxyl terminus of STAT6 contains a modular transcription activation domain that does not need IL-4 for its activation function.

To further define the STAT6 transcriptional activation domain, a series of constructs in which different portions of the carboxyl region of murine STAT6 fused to Gal4 DNA binding domain were generated (Fig. 1*B*) and assayed in NIH3T3 cells. A truncation mutant Gal4-ST6 (726–837) that lacks 130 aa from the amino terminus of the initial (aa 596–837) construct still possesses full activating properties in these cells, while a mutant that lacks an additional 59-aa Gal4-ST6 (785–837) does not possess activating properties. Similarly, assays using a carboxyl-terminal truncation mutant Gal4-ST6 (596–785) revealed that this fusion protein retains full scale *trans*-activation in this cell line, while a mutant that lacks an additional 59 aa from the carboxyl terminus Gal4-ST6 (596–726) has greatly reduced activity in this assay. These fusion proteins suggest that a minimal transcriptional activity domain might be present in the region of STAT6 in which they overlap (aa 726–785). However, a construct using only this overlapping region Gal4-ST6 (726–785) does not activate transcription in this assay (Fig. 1*C*). The failure of this region of STAT6 to act as an activating domain suggests that there might be two overlapping regions of STAT6 that can function to activate transcription. The analysis of two additional Gal4-STAT6 fusion proteins in NIH3T3 cells maps the minimal core transcriptional activating region of STAT6 to aa 656–785.

To further analyze the sequences required for the transcriptional activating function of the carboxyl region of STAT6, the properties of Gal4-STAT6 fusion proteins were also assayed in the murine M12.4.1 B cell line. Expression of the Gal4-STAT6 fusion protein that contains the entire murine STAT6 carboxyl region (aa 596–837) greatly induces reporter expression in these cells (Fig. 1*C*). In contrast to the results in the NIH3T3 cells (which showed that the STAT6 carboxyl region was one-third as potent as VP16), the levels of reporter expression induced by this Gal4-STAT6 fusion protein are higher than the levels induced by the Gal4-VP16 in the M12.4.1 cells. This suggests that this region of STAT6 can act as an extremely potent transcriptional activator in this murine B cell line.

To further map the domain of STAT6 required to activate transcription, other Gal4-STAT6 fusion proteins were also examined in this assay system. The fusion proteins that lack transcriptional activating activity in NIH3T3 cells (Gal4-ST6 (785–837), Gal4-ST6 (726–785), and Gal4-ST6 (596–726)) also fail to generate significant transcriptional activation in M12.4.1 cells. Interestingly, the Gal4-ST6 (726–837) and Gal4-ST6 (596–785), which are as potent as Gal4-ST6 (596–837) in the assays performed in NIH3T3 cells, possess significantly less transcriptional activation activity in M12.4.1 cells. Consistent with this result, Gal4-ST6 (645–785) and Gal4-ST6 (656–785) also produced less transcription activation than the Gal4-ST6 (596–837) in these cells. Therefore, the whole carboxyl region (aa 596–837) of STAT6 is required to achieve maximal *trans*-activation in this murine B cell line. These results indicate that there is a difference in activity of these Gal4-STAT6 fusion proteins when assayed in the M12.4.1 B cell line and the NIH3T3 fibroblast cell line, and suggest that the STAT6 transcription activation domain may function differently in these two cell lines.

The carboxyl-terminal region of STAT6 is required for IL-4 induction of transcription

The experiments using the Gal4-STAT6 fusion proteins support the hypothesis that the carboxyl terminus of STAT6 contains a potent transcriptional activation domain. To determine whether this domain is required for IL-4-induced transcriptional activation mediated by STAT6, two truncation mutants of the full-length STAT6 were generated using a HuSTAT6 cDNA. One truncation mutation, which removes aa 804–847 from the HuSTAT6 protein (which corresponds to aa 795–837 of the mouse protein), leaves most of the putative *trans*-activation domain intact. A second truncation mutation, which removes aa 659–847 from the protein (corresponding to murine aa 659–837), deletes the entire putative *trans*-activation domain while leaving the tyrosine residue (aa 641) required for STAT6 dimerization intact (see Fig. 2*A*). These cDNA were cloned into a CMV promoter-based expression vector pcDNA3.

To analyze the properties of these STAT6 mutants, a reporter construct was generated in which the C/EBP/STAT6 binding site from the murine Ig germline Iε promoter was multimerized (a tetramer) and cloned upstream of a minimal TK promoter and a luciferase reporter gene (termed $p(I\epsilon\text{-IL4RE})_4$-Luc). This STAT6 binding site is required for the IL-4-mediated induction of transcription from the Iε promoter (23). This reporter construct was cotransfected with the different STAT6 expression vectors into the human 293 cell line. After transfection, cells were split into two equal populations, and the cells were either grown in media alone or with IL-4 added. Cells were harvested after 36 h, and extracts were assayed for luciferase activity (see Fig. 2*B*). In some experiments, extracts harvested from these cells were assayed for STAT6 activation by EMSA (see Fig. 2*C*).

When 293 cells are cotransfected with the $p(I\epsilon\text{-IL4RE})_4$-Luc reporter and an empty pcDNA3 expression vector, there is almost no luciferase activity (above baseline) if cells are cultured alone or with IL-4. Cells cotransfected with the reporter and the full-length STAT6 expression vector contain low levels of luciferase activity. However, when cells cotransfected with the full-length STAT6 expression vector and the reporter plasmid are cultured with IL-4,

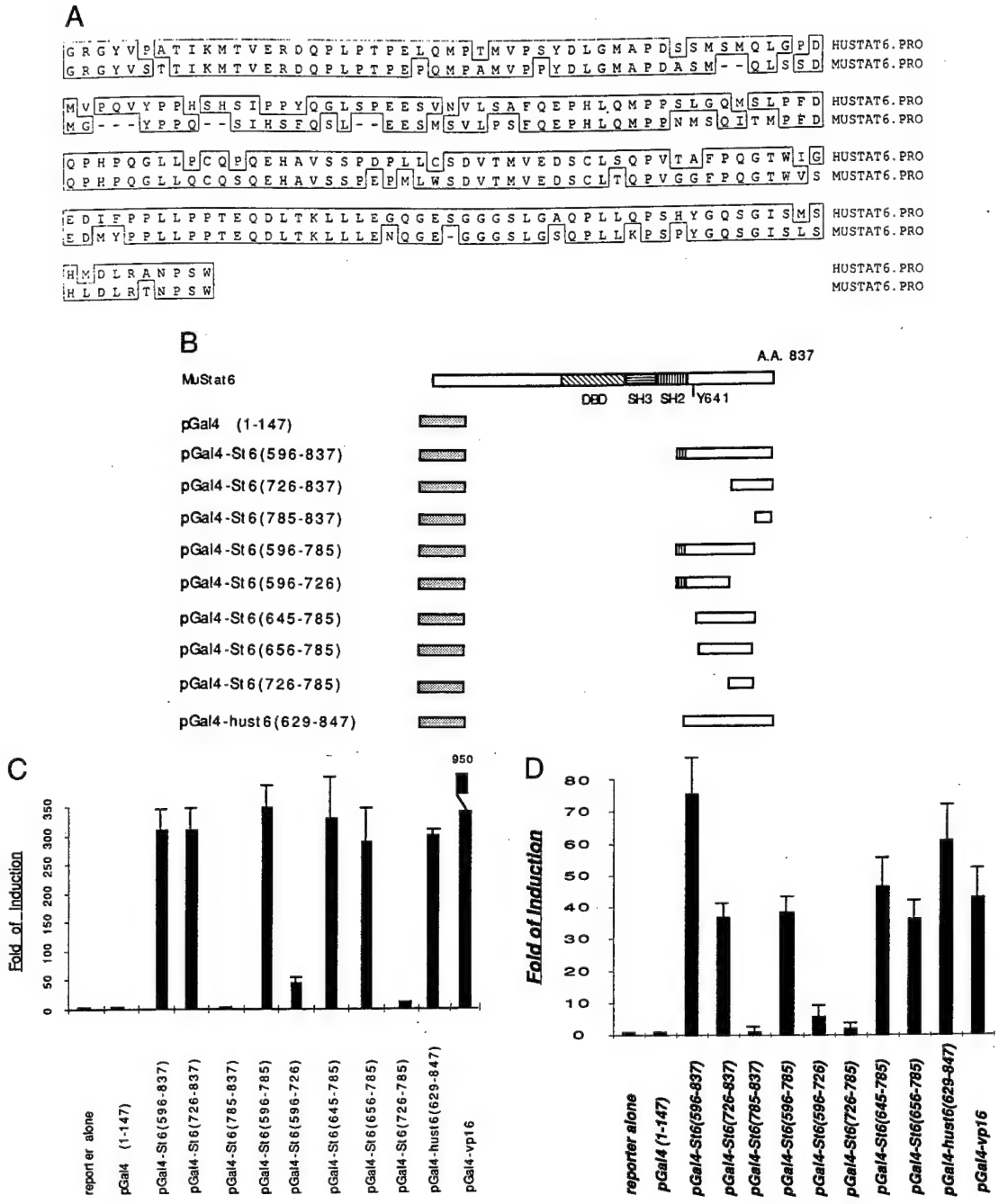


FIGURE 1. Identification of a transcriptional activation domain in the carboxyl terminus of STAT6 using Gal4-STAT6 chimeric proteins. *A*, Comparison of the peptide composition of the carboxyl regions of murine STAT6 (GenBank accession number P52633) and HuSTAT6 (GenBank accession number P42226) proteins. *B*, Schematic drawing of Gal4(1–147) fusion constructs. DBD is the DNA binding domain. Y641 is the tyrosine that is phosphorylated when STAT6 is activated. Amino acid 837 is the last amino acid of murine STAT6. Gal4-huSt6 (629–847) is the fusion protein of Gal4(1–147) and HuSTAT6 from aa 629 to aa 847. All of the rest of the constructs express fusion proteins composed of the Gal4 DNA binding domain (1–147) and the indicated amino acids of murine STAT6 protein. *C, trans*-activation activity of different Gal4(1–147)-STAT6 fusion proteins in NIH3T3 cells. NIH3T3 cells were transfected by calcium phosphate precipitation. Two micrograms of each Gal4(1–147) fusion constructs were cotransfected with 1 μg pGal$_5$-Luc reporter plasmid and 1 μg pSV40-βgal plasmid. Thirty-six hours after transfection, the cells were harvested and the luciferase activity was determined. Luciferase values were then adjusted for the transfection efficiency by standardizing with the β-gal activity of each sample. The fold of induction is calculated by $(LUC_{gal4\text{-}St6} - LUC_{gal4})/LUC_{gal4}$, in which $LUC_{gal4\text{-}St6}$ represents the normalized luciferase activities of extracts made from cells transfected with the individual Gal4-STAT6 fusion plasmid and LUC_{gal4} represents the luciferase activity of the extract made from cells transfected with Gal4(1–147) plasmid. The results shown are the average of six independent transfections. *D, trans*-activation activity of Gal4(1–147) fusion proteins in M12.4.1 cells. M12.4.1 cells were transfected as described in *Materials and Methods*. The fold of induction is calculated as described above for NIH3T3 cells. The results shown are the average of six transfections.

there is a 250-fold induction in luciferase activity (Fig. 2). Analysis of cells transfected with the vector expressing the HuSTAT6-Δ(804–847) mutant reveals that this protein endows these cells with IL-4-inducible (98-fold) expression of the luciferase reporter. However, the level of induction is somewhat reduced (by 60%) when compared with the full-length STAT6. These results contrast

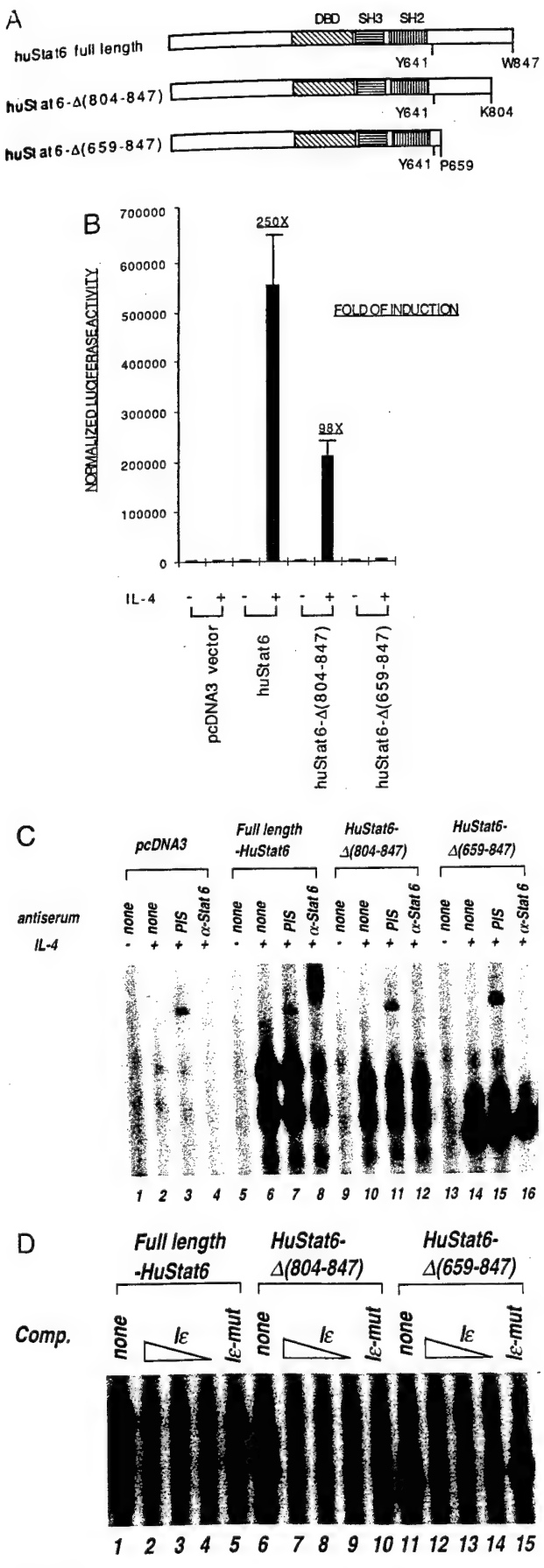


FIGURE 2. The effects of two carboxyl-terminal truncations of STAT6 on IL-4-induced transcription. *A*, Schematic drawing of full-length HuSTAT6 and two truncation mutants. The numbers and identity of the last amino acid in each STAT6 protein are marked, which are W847, K804, and P659. *B*, Gradual loss of transcriptional activation activity by serial carboxyl truncation of STAT6 protein. Each of these expression constructs is cotransfected with 1 μg p(Iε-IL4RE)$_4$-Luc and 1 μp-SV40-βGal into 293 cells. Twenty-four hours after transfection, cells are treated with human IL-4 for 12 h. Cells are harvested and luciferase activity is measured with standardized to β-gal activity. The fold of induction is calculated by the formula ($LUC_{IL-4} - LUC_{none}$)/LUC_{none}, in which LUC_{IL-4} stands for luciferase activity after IL-4 treatment and LUC_{none} stands for luciferase activity without IL-4 treatment. *C*, DNA-binding complexes formed in cells transfected with STAT6 expression vectors. EMSA analysis of IRF-1 GAS-binding activity in 293 cells either before (−) or 15 min after (+) culture with IL-4, *Lanes 1* to *4*, Extract from 293 cells transfected with empty vector; *lanes 5* to *8*, extract from 293 cells transfected with STAT6 expression vector; *lanes 9* to *12*, extract from 293 cells transfected with STAT6-Δ(804-847); and *lanes 13* to *16*, extract from 293 cells transfected with STAT6-Δ(659-847) expression plasmids. EMSA reactions were assayed for the presence of STAT6-dependent complexes by coincubation either with preimmune sera (*lanes 3, 7, 11*, and *15*) or α-STAT6 antisera M-20 (*lanes 4, 8, 12*, and *16*). *D*, STAT6 and STAT6 mutants have similar binding affinities for different STAT-binding elements. The binding of STAT6 to IRF-1 GAS element (*lanes 1, 6*, and *11*) was competed by nonlabeled oligonucleotides representing either the binding site from the murine germline ε promoter (Iε) (*lanes 2, 3, 4, 7, 8, 9, 12, 13*, and *14*) or mutant Iε probe (*lanes 5, 10*, and *15*). Each EMSA reaction contains 2 ng of radioactively labeled oligonucleotide. Total amount of cold wild-type competitors is 10 ng (*lanes 4, 9*, and *14*), 33 ng (*lanes 3, 8*, and *13*), or 100 ng (*lanes 2, 7*, and *12*). The amount of mutant competitor is 100 ng (*lanes 5, 10*, and *15*).

with experiments utilizing the HuSTAT6-Δ(659–847) mutant, which lacks the entire putative *trans*-activating domain. When this truncated protein is expressed in 293 cells, it fails to allow cells to activate transcription from the pI$_\epsilon$-Luc reporter after IL-4 treatment. These experiments suggest that the carboxyl terminus of STAT6 is required for IL-4-inducible transcription.

Truncated STAT6 proteins can form DNA-binding complexes

The transfection experiments in 293 cells illustrate that the carboxyl-terminal region of STAT6 is required for IL-4 induction of transcription from a STAT6 reporter construct. To further understand the mechanism underlying this effect, we assayed whole cell extracts isolated from transfected 293 cells for STAT/DNA-binding activity by EMSA with the IRF-1 GAS probe that binds STAT6 (12). These complexes were also examined by a STAT6-specific antisera M-20. The anti-STAT6 antiserum was generated against a peptide (aa 805–823) of murine STAT6 protein, which is a region highly conserved in HuSTAT6. Therefore, the antisera should affect EMSA bands that contain full-length STAT6 protein without altering bands formed solely by the deletion mutants.

When 293 cells transfected with the empty expression vector are analyzed, IL-4 induces a very small amount of GAS-binding activity (Fig. 2*C*, *lanes 1–4*). These GAS-binding complexes contain STAT6 as they are supershifted by the STAT6-specific antiserum (Fig. 2*C*, *lane 4*). Interestingly, cells transfected with the full-length STAT6 expression vector exhibit a large increase in the levels of these same DNA-binding complexes (*lanes 5–8*). These complexes are also supershifted with the α-STAT6 sera (*lane 8*). When extracts from cells transfected with the HuSTAT6 mutant Δ(804–847) expression vector are analyzed, they also contain IL-4-induced GAS-binding complexes. When compared with complexes generated by transfection of 293 cells with the full-length STAT6 expression vector, the complexes generated with the HuSTAT6-Δ(804–847) vector possess a slightly faster mobility on EMSA, consistent with a smaller STAT6 protein. The inducible

EMSA bands in 293 cells transfected with the HuSTAT6-Δ(804–847) vector do not appear to be decreased/ablated by the α-STAT6 sera. EMSA studies of cells transfected with the HuSTAT6-Δ(659–847) expression vector show that this truncated protein can form DNA-binding complexes when these cells are cultured with IL-4 (*lanes 13–16*). There are three IL-4-inducible EMSA bands in 293 cells transfected with STAT6-Δ(658–847). The two slower migrating bands are decreased when the binding reactions are incubated with the anti-STAT6 antiserum. The slower of these bands migrates with the same mobility as one of the bands observed in untransfected 293 cells (see *lane 2*) and most likely represent the activated form of the endogenous STAT6. Because the HuSTAT6-Δ(659–847) mutant does not contain the residues recognized by this antiserum, it is possible that the faster one of these two bands is formed by a heterodimer between the mutant and wild-type STAT6.

To determine whether the complexes formed by these mutant STAT6 molecules bind DNA with high affinity, we examined the ability of different amounts of nonlabeled oligonucleotide to compete the IRF-1 GAS probe for binding to STAT6 (Fig. 2*D*). These EMSA show that DNA-binding complexes formed by the wild-type HuSTAT6 and both truncated forms of STAT6 expressed in 293 cells are extremely sensitive to competition by unlabeled IRF-1 GAS oligonucleotide. In addition, these complexes are all poorly competed by a mutated STAT6 binding site. These EMSA suggest that the inability of the mutant STAT6 molecules to activate transcription does not result from changes in their DNA-binding properties, and support the idea that the loss of function imparted by these truncation mutations results from the loss of a region required for transcriptional activation.

Increased levels of STAT6 enhance the ability of IL-4 to induce transcription in 293 cells

In 293 cells, IL-4 induces an extremely low level little GAS-binding activity. In addition, IL-4 does not induce transcription from the STAT6-responsive reporter in these cells. In contrast, IL-4 induces a significant GAS-binding activity in 293 cells transfected with the full-length STAT6 expression vector. This correlates with the induction of transcription from the STAT6-responsive reporter in these cells, and suggests that STAT6 might be a limiting factor for IL-4-induced transcription in these cells. To further examine this possibility, cotransfection experiments were performed to determine whether increasing expression of STAT6 alters IL-4-mediated transcriptional activation in 293 cells in a dose-dependent manner. 293 cells were cotransfected with the p(Iε-IL4RE)$_4$-Luc reporter and increasing amounts of the full-length STAT6 expression vector and the ability of IL-4 to induce the reporter were measured by luciferase activity (Fig. 3*A*). These experiments show a strong correlation between the IL-4 induction of the reporter and the amount of the STAT6 expression vector cotransfected. These data support the hypothesis that STAT6 levels might limit the IL-4 responsiveness of these cells.

Expression of truncated STAT6 inhibits IL-4-induced transcription

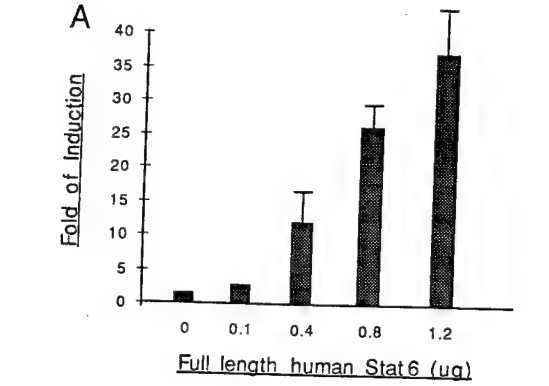
The HuSTAT6-Δ(659–847) loses the ability to convey IL-4-activated transcription from a p(Iε-IL4RE)$_4$-Luc reporter plasmid, yet this protein can still be activated to form GAS-binding complexes by IL-4. This suggests that this truncation mutant might be able to suppress IL-4-induced transcription mediated by a wild-type STAT6. To address this question, we exploited the 293 transient transfection system to overexpress different amounts of STAT6 proteins and determine whether the truncated STAT6 protein can act as a dominant negative mutant. A fixed amount of full-length HuSTAT6 expression plasmid (0.8 μg) and p(Iε-IL4RE)$_4$-Luc reporter construct (0.8 μg) were cotransfected with increasing amounts of the HuSTAT6-Δ(659–847) expression plasmid DNA into 293 cells. The total amount of DNA in each individual transfection was equalized by adding the empty expression vector pcDNA3 DNA. As observed previously, a significant stimulation of IL-4-induced reporter luciferase activity (26-fold) is observed when the full-length HuSTAT6 expression vector with pcDNA3 vector is transfected. When increasing amounts of the HuSTAT6-Δ(659–847) mutant are expressed in these cells, the IL-4-induced luciferase activity decreases in a dose-dependent manner (Fig. 3). These data indicate that the HuSTAT6-Δ(659–847) can act as dominant negative mutant in 293 cells.

trans-activation domain-deleted mutant STAT6 can specifically block IL-4-induced gene expression from a natural promoter

The studies above examine the function of STAT6 truncation mutants using an artificial promoter system in which multimerized STAT binding sites are placed upstream of a minimal promoter. Recent data suggest that the mechanism by which STATs induce transcription might differ when similar artificial promoter systems are compared with native promoters (18). In addition, *trans*-activation domains have been shown to function in a context-dependent manner (37). We therefore examined the ability of these STAT6 truncation mutant proteins to affect the transcription induced from IL-4-responsive promoters/genes.

It has been shown previously that IL-4 can induce transcription from the Ig germline ε promoter in the human B cell lymphoma line DND39/L12. To facilitate a quantitative analysis of the expression of germline ε transcripts, this cell line was also stably transfected with a luciferase reporter construct under control of the germline ε promoter. This promoter is IL-4 inducible and it contains two STAT6 binding sites, one of which is essential for IL-4 inducibility (20, 23). An expression vector that contained the cDNA encoding the HuSTAT6-Δ(659–847) mutant under the control of the inducible metallothionine promoter was stably transfected into these cells. Transcription from this metallothionine promoter can be induced by metals such as $CdCl_2$. As a control, we also generated stable lines of DND39/L12 cells transfected with the empty metallothionine promoter expression vector (pMEP-4). Western blotting reveals that when the cells transfected with the HuSTAT6-Δ(659–847) metallothionine expression cassette are cultured with 1 mM of $CdCl_2$ for 24 h, high levels of the mutant HuSTAT6-Δ(659–847) protein are expressed (Fig. 4*A*). The optimal time point for induction of the mutant HuSTAT6-Δ(659–847) protein is 48 h (data not shown). Furthermore, EMSA with the IRF-1 GAS probe demonstrates that this truncated protein can be activated, in response to IL-4, to form DNA-binding complexes in these cells (Fig. 4*B*).

The inducible expression of the mutant HuSTAT6-Δ(659–847) protein in DND39/L12 cells establishes a system to determine whether expression of this mutant protein affects transcription from the Ig germline ε promoter. DND39/L12 transfected with either the pMEP-4 vector alone or the vector with the mutant HuSTAT6-Δ(659–847) were induced with $CdCl_2$ at 1 mM for 48 h, and then treated with human IL-4 overnight and assayed for luciferase activity generated from the reporter. Wild-type DND39/L12 cells or cells transfected with the vector pMEP-4 express extremely low levels of luciferase when grown in culture. When these cells are cultured overnight with human IL-4, luciferase activity is greatly induced (Fig. 4*C*). Strikingly, when cells expressing the mutant HuSTAT6-Δ(659–847) are examined, the IL-4 induction of the germline ε reporter is greatly reduced. (Fig. 4*C*).



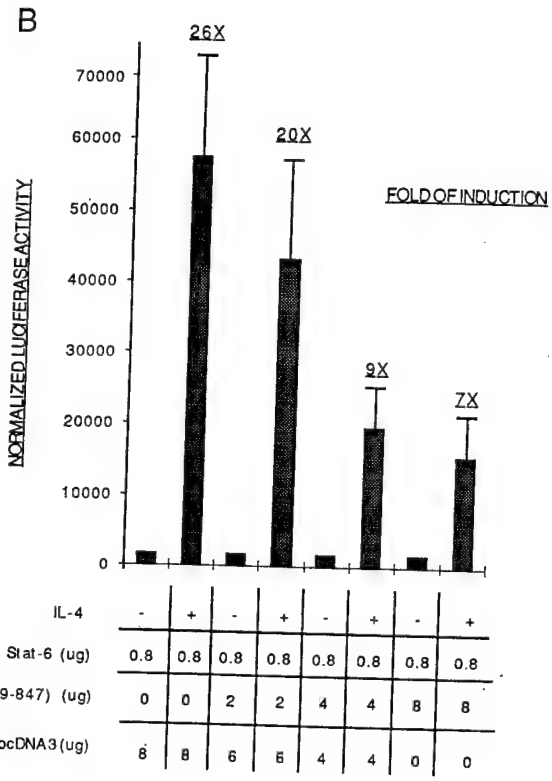


FIGURE 3. The effects of overexpression of STAT6 and STAT6-Δ(659–847) on IL-4-induced transcriptional activation. *A*, Overexpression of full-length STAT6 proteins increases IL-4-induced transcription activation in dosage-dependent manner. 293 cells are transfected with 0.8 μg of p(Iε-IL4RE)$_4$-Luc reporter plasmids plus increased amount of full-length HuSTAT6 expression plasmids. βGal expression plasmids are cotransfected for standardization of different transient transfection. Twenty-four hours after transfection, the cells are cultured with IL-4 for 12 h. Cells are harvested and luciferase activities are determined. The luciferase activities are standardized to βgal activities. *B*, Dominant negative effect of HuSTAT6-Δ(659–847) on IL-4-induced transcriptional activation. 293 cells were cotransfected with 1 μg of the p(Iε-IL4RE)$_4$-Luc repoter plasmid, 0.8 μg of the full-length HuSTAT6 expression plasmid, and increased amount of HuSTAT6-Δ(659–847). The luciferase activities are standardized as described above. The fold of induction of transcription by IL-4 is described above.

These data indicate that the mutant STAT6 can inhibit the IL-4 induction of transcription initiating from the germline ε promoter.

To determine whether STAT6 is required for the IL-4-induced transcription of the endogenous germline ε gene, we examined the level of these RNA. RNA was isolated from cells cultured with IL-4 for 3 days and Northern blotted with a human Cε probe. When compared with the control cells (transfected with the expression vector alone), the level of IL-4 germline ε transcripts induced by IL-4 is decreased in the cells overexpressing the mutant HuSTAT6-Δ(659–847) protein (Fig. 4*D*). This result supports the data from experiments examining the Iε-Luc reporter construct in these cells and indicates that this mutant form of STAT6 can inhibit the IL-4 induction of the germline ε promoter.

Discussion

Our results demonstrate that STAT6 contains a modular, proline-rich *trans*-activation domain. Proline-rich domains have been found in a number of transcription factors that can either activate or repress transcription, such as BSAP (38) and EKLF (34). Although the mechanism by which these domains function is still not clear, proline-rich domains have been shown to recruit downstream basal level transcription factors such as TBP (39), and to reorganize the chromatin structure through interaction with histone 3 (40). The carboxyl region of STAT6 protein may use a similar mechanism to activate IL-4-induced transcription. Interestingly, when different Gal4-STAT6 fusion constructs were assayed in NIH3T3 and M12.4.1, they displayed a different pattern of transcriptional activation abilities. In contrast to results in the NIH3T3 cells, in which a truncated core region of STAT6 was sufficient for maximal transcriptional activation, the data in M12.4.1 cells demonstrated that any truncation of the carboxyl region of STAT6 reduces the transcriptional activation activity in the context of the Gal4-STAT6 fusion proteins. This result is consistent with the experiments in which the transcriptional activity is examined in the context of the STAT6 protein in 293 cells, which demonstrate that a small truncation at the carboxyl terminus of STAT6 decreases the protein's ability to activate transcription and suggests that, in some cells, this entire region of STAT6 (aa 596–837) is required to achieve maximal transcription activation. The different relative transcriptional activation activities of the Gal4-STAT6 and Gal4-VP16 fusion proteins observed when NIH3T3 cells and M12.4.1 cells are compared also suggest that there may be cell-specific influences on the function of STAT6.

There are several possible mechanisms by which the activation domain of STAT6 functions differently in various cell types. One possibility is that the transcriptional cofactors recruited by the domain may be different in these two cell lines. It has been reported that there are cell type-specific transcription cofactors in differentiated B cells (41). The difference in the pool of cofactors in different cell types may affect the function of *trans*-activation domain. Alternatively, it is possible that different post-translational modification of residues within these domains occurs in these cell lines, and that these modifications alter the activity of these proteins (42, 43).

Post-translational modifications of *trans*-activation domains, such as serine/threonine phosphorylation, have been shown to be responsible for the *trans*-activation potency of a number of transcription factors, including STAT1 and STAT3 (44). This phosphorylation may involve the activation of serine/threonine kinases through the mitogen-activated protein kinase (MAPK) pathway. Interestingly, IL-4 does not activate *ras*/MAPK pathway in most of cell types (45). In addition, there are no consensus MAPK phosphorylation sites in the carboxyl domain of STAT6. This is consistent with our observation that IL-4 treatment does not seem to increase the *trans*-activation potency of Gal4-STAT6 fusion proteins in either NIH3T3 cells or M12 cells. It has been shown previously that the carboxyl terminus of STAT2, STAT3, and STAT5 contains transcriptional activating domains. Our data demonstrate

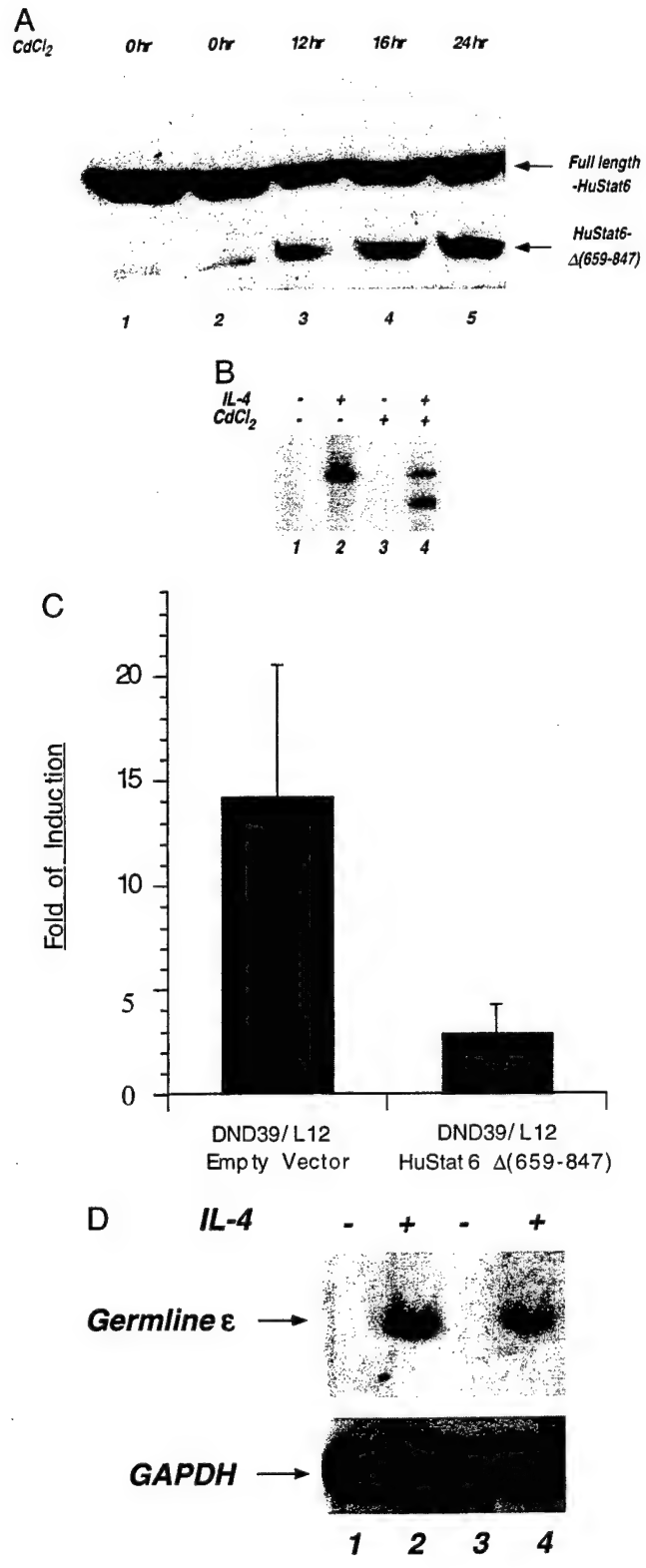


FIGURE 4. The STAT6-Δ(659–847) protein suppresses IL-4-induced transcriptional activation of Ig germline ϵ (Iϵ) promoter. *A*, Time course of $CdCl_2$ induced expression of HuSTAT6-Δ(659–847). DND39/L12 cells stably transfected with STAT6-Δ(659–847) expression plasmids, as described in *Materials and Methods*. Cells are kept in 400 μg/ml hygromycin-B and 1 mM $CdCl_2$ for different time points, as indicated. Western blot is done with anti-HuSTAT6 antisera 11503, which are generated against SH2 domain of HuSTAT6 protein. *B*, The HuSTAT6-Δ(659–847) protein is induced to form IRF-1 GAS-binding complexes by IL-4. Both DND39/L12 cells stably transfected with the empty vector and DND39/L12 cells stably transfected with the STAT6-Δ(659–847) expression vector are cultured with hygromycin-B at 400 μg/ml and 1 mM $CdCl_2$ for 24 h. Then they are treated with human IL-4 for 15 min. Whole cell extracts are assayed for GAS DNA-binding complexes by EMSA. *Lane 1*, Extracts of DND39/L12 cells transfected with the empty vector. *Lane 2*, Extracts of DND39/L12 cells transfected with the empty vector treated with IL-4 for 15 min. *Lane 3*, Extracts of DND39/L12 cells transfected with the STAT6-Δ(659–847) expression vector. *Lane 4*, Extracts of DND39/L12 cells transfected with the STAT6-Δ(659–847) expression vector treated with IL-4 for 15 min. *C*, The HuSTAT6-Δ(659–847) protein suppresses IL-4-induced luciferase activity from I_ϵ-Luc reporter. DND39/L12 cells are stably transfected with either the empty vector pMEP-4 alone or pMEP-4-HuSTAT6-Δ(659–847). The cells are induced by 1 mM of $CdCl_2$ for 48 h and cells are counted. Two million cells were put in each 60-mm TC plate and culture with or without IL-4 overnight. The luciferase fold of induction is calculated by dividing the luciferase activity of the extracts of cells treated with IL-4 by that of the extracts of untreated cells. The activity from I_ϵ-Luc reporter. DND39/L12 cells are stably transfected with either the empty vector pMEP-4 alone or pMEP-4-HuSTAT6-Δ(659–847). The cells are induced by 1 mM of $CdCl_2$ for 48 h and cells are counted. Two million cells were put in each 60-mm TC plate and culture with or without IL-4 overnight. The luciferase fold of induction is calculated by dividing the luciferase activity of the extracts of cells treated with IL-4 by that of the extracts of untreated cells. The luciferase fold of induction is the average of five experiments. *D*, The HuSTAT6-Δ(659–847) protein suppresses IL-4-induced germline ϵ message. DND39/L12 cells are stably transfected with either the empty vector pMEP-4 or pMEP-4-HuSTAT6-Δ(659–847). The cells are induced by 1 mM of $CdCl_2$ for 24 h, and either treated with IL-4 or left untreated for 3 days.

that the carboxyl terminus of STAT6 also contains a transcriptional activating domain. It is interesting that the peptide composition of the carboxyl terminus of STAT6 is quite different from that of other members of the STAT family. This suggests that, although structurally the transcriptional activating domain of STATs may need to be located at the carboxyl terminus of the protein, different STATs may use different mechanisms (i.e., cofactors) to activate transcription.

The ability of cells to respond to cytokines can potentially be regulated at several different levels. One common point of controlling cytokine responsiveness is to regulate the surface expression of cytokine receptors (7). Another mechanism used to regulate cytokine responsiveness may be to modulate the levels of downstream signaling molecules. For example, both type 1 and type 2 IFNs can increase the expression of STAT1 (17). We have observed that the level of STAT6 protein varies in different cell lines. The human kidney cell line 293 expresses barely detectable levels of endogenous STAT6 protein. Although IL-4 can activate STAT6 in 293 cells, it fails to induce expression of the reporter gene. In contrast, DND39/L12 cells express high level of STAT6 protein and this same reporter construct can be induced by IL-4 in these cells. By overexpressing STAT6 protein in 293 cells, we reconstitute the IL-4 responsiveness in these cells. This demonstrates that STAT6 is the limiting factor for IL-4-induced gene expression in 293 cells and illustrates a new level at which the cellular response to IL-4 can be controlled.

The data above analyzing mutant STAT6 proteins in 293 cells and DND39/L12 cells demonstrate that the carboxy-deleted mutants both lose the ability to transcriptionally activate the IL-4-responsive reporter genes and also block the IL-4-induced transcription of the germline ϵ locus. Although expression of the

truncated STAT6 greatly reduced the levels of germline ϵ transcripts induced by IL-4, it does not completely eliminate the induction of these mRNA. It is likely that the high levels of endogenous STAT6 protein expressed by these cells (Fig. 4A) dampen any dominant effect of the truncated protein. The induction of germline ϵ transcripts in response to IL-4 is essential for Ig class switching to IgE. The failure of STAT6-deficient mice to produce IgE in response to IL-4 has been presumed to result from the inability to induce these transcripts. The results presented in this work support this model and directly demonstrate that STAT6 is important for the induction of germline ϵ transcripts by IL-4.

Expression of the carboxyl deletion mutant STAT6 may block IL-4-induced transcription by several different possible mechanisms. First, it could compete with wild-type STAT6 for binding to the IL-4R that, after it is activated by tyrosine phosphorylation, has several docking sites for STAT6. Second, it could form both heterodimer with full-length STAT6 and homodimer by itself and compete for machinery for nuclear import. Third, the homodimer of the truncation mutant can compete for DNA binding sites with the wild-type STAT6. Fourth, it is likely the heterodimer between full-length STAT6 and carboxyl-truncated STAT6 has less transcriptional activation activity than the wild-type STAT6. EMSA analysis of the extract of cells transfected with STAT6-Δ(658–847) does suggest that both heterodimers and homodimers may be present in these cells. Because both tyrosine 641 and the SH2 domain, which are the regions involved in dimer formation, are present in this mutant, IL-4 could activate both mutant and wild-type forms of STAT6 in these cells and allow the formation of heterodimers and homodimers of these proteins. However, we have been unable to confirm the presence of these heterodimers using immunoprecipitation experiments examining 293 cells transfected with the STAT6-Δ(658–847) expression vector (B. Lu and P. B. Rothman, unpublished data).

It is worth mentioning that during the preparation of this manuscript, another group also demonstrated the role of the carboxyl region of STAT6 using a similar approach (46). In addition, in this work, they use a reporter construct with combined C/EBP and STAT6 binding sites taken from Cϵ promoter that is similar to our reporter plasmid. After mutating either C/EBP or STAT6 binding sites, the reporter gene fails to be induced by IL-4. This confirms the previous study that mutation of this site within the context of an intact promoter results in loss of IL-4 inducibility of the germline ϵ promoter in M12.4.1 cells (23). These results suggest that both STAT6 and C/EBP sites are essential for IL-4-induced transcription, although only the STAT6 site is required for binding of STAT6 in vitro. We also observed lack of response to IL-4 when the STAT6 sites alone are multimerized, inserted into the same minimal promoter, and transfected into murine B cells (data not shown). In addition, the C/EBP site is found to be close to the STAT6 binding site in several natural IL-4-inducible promoters, such as CD23, Ig Cϵ, and Ig Cγ1 (23). This implies that, in order for STAT6 to function properly as a transcriptional activator, a C/EBP site-binding factor must be present nearby. It is interesting that the STAT6 transcriptional activation domain can function properly when fused to Gal4 DNA binding domain, but fails to do so in the context of natural STAT6 protein when only STAT6 sites are present. Although the C/EBP site was shown previously to be important for the IL-4 response mediated by this element and C/EBP family proteins have been shown to bind to this site in M12.4.1 cells (23), overexpression in 293 cells of either C/EBP-β or C/EBP-α does not act synergistically with STAT6 to activate this reporter (B. Lu and P. B. Rothman, data not shown). Contrary to the essential role of C/EBP site for the IL-4-induced transcription activation, there is only a half C/EBP site present on a fully functional murine germline ϵ promoter (20). These observations argue against the positive role that C/EBP family members may play in this system.

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The IL-4 Receptor α-Chain Cytoplasmic Domain Is Sufficient for Activation of JAK-1 and STAT6 and the Induction of IL-4-Specific Gene Expression[1]

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The common γ-chain (γ_c) is a functional component of the IL-4R, yet cells lacking γ_c are able to respond to IL-4. This has led to the suggestion that a surrogate γ'-chain, which can interact with the IL-4Rα chain to mediate signaling, is expressed on cells lacking γ_c. An alternative possibility is that in the absence of γ_c, the IL-4Rα chain is able to transduce signals by homodimerization. To test this latter possibility, a chimeric receptor containing the extracellular domain of c-*kit* (the stem cell factor (SCF) receptor) and the cytoplasmic and transmembrane domains of the IL-4Rα chain was generated. Treatment of cells expressing the chimeric receptor kit/IL-4Rα with SCF induces activation of the IL-4Rα-associated kinase JAK-1 and the transcription factor STAT6. However, tyrosine phosphorylation of JAK-3, which associates with γ_c, is not induced by SCF in these cells. SCF-mediated ligation of kit/IL-4Rα is sufficient to elicit IL-4-specific gene expression, including up-regulation of CD23 and synthesis of germ-line ϵ transcripts. In the T cell line CTLL2, ligation of kit/IL-4Rα induces cellular proliferation. Finally, in JAK-1-deficient HeLa cells, STAT6 activation by IL-4 is completely abolished. Together, these data demonstrate that the IL-4Rα cytoplasmic domain is sufficient to activate JAK-1 and STAT6 and to induce expression of IL-4 target genes, thus identifying a mechanism by which IL-4 signaling can proceed in the absence of JAK-3 and γ_c. *The Journal of Immunology,* **1997, 158: 5860–5867.**

An emerging principle for the initiation of signal transduction by diverse classes of transmembrane receptors is the requirement for ligand-induced oligomerization of receptor subunits. For several cytokines, e.g., growth hormone and IL-6, a single receptor component undergoes homodimerization to initiate downstream signaling (1, 2). In contrast, other cytokines activate signaling cascades through the activation of two distinct receptor chains. For example, IFN-γ signals through heterodimerization of the IFN-γR α-chain and β-chain (3).

Until recently, the cell surface receptor for IL-4 was thought to consist of a single chain, IL-4Rα, because this chain alone is capable of high affinity binding of ligand (4). However, the γ-chain of the IL-2R (termed γ_c)[4] was subsequently shown to participate in IL-4R signaling in multiple cell types. For example, COS-7 cells transfected with IL-4Rα and γ_c had three- to fourfold higher affinity for IL-4 than cells expressing IL-4Rα alone, suggesting that γ_c plays a role in ligand binding (5).

Treatment of cells with IL-4 induces rapid tyrosine phosphorylation of multiple cellular proteins, including the receptor itself. This phosphorylation is conducted by members of the receptor-associated Janus kinase (JAK) family of protein tyrosine kinases. JAK-1 associates with the IL-4Rα chain, and JAK-3 associates with the γ_c chain. Upon receptor-mediated activation, JAK-1 and JAK-3 are thought to phosphorylate specific tyrosine residues on the cytoplasmic domain of the IL-4Rα chain, which thereafter serve as docking sites for downstream signaling molecules. Among these signaling molecules is STAT6, a member of the STAT family of transcription factors. Thereupon, tyrosine phosphorylation of STAT6 ensues, permitting homodimerization and translocation of this complex to the nucleus. STAT6 binding sites have been shown to be functionally important for the IL-4 induction of several genes (6–9).

Although the IL-2 and IL-4 signal transduction cascades both use the γ_c as part of their respective receptor complexes and both activate JAK-1 and JAK-3, these cytokines induce distinctive biological activities (5, 10–15). For example, IL-4 induces the activation of STAT6 and c-*fes*, whereas IL-2 activates STAT5 and members of the *src* family of kinases, such as *lck* (16–18). The activation of these various signaling pathways leads to the transcription of different genes. For example, STAT6 binding sites have been found within the promoters of several IL-4-inducible genes, including CD23, MHC class II, Iϵ, and Iγ1 (8, 19–22). Interestingly, STAT6-deficient mice are not able to induce expression of CD23, MHC class II, or synthesize IgE in response to IL-4 (23–25).

Recent studies have defined regions of the cytoplasmic domain of IL-4Rα that mediate specific aspects of IL-4 signaling. Several IL-4-specific functions can be transferred to the IL-2R by transplanting tyrosine-containing sequences from the cytoplasmic domain of IL-4Rα (26). The region between amino acids 557 and 657 of IL-4Rα has been shown by mutational analysis to be required

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[4] Abbreviations used in this paper: γ_c, γ-chain of the IL-2R; JAK, Janus kinase; SCF, stem cell factor; EMSA, electrophoretic mobility shift assay; IP, immunoprecipitation; GAS, interferon-γ-activating sequences.

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for full expression of IL-4-responsive genes (27). Furthermore, at least one of the three tyrosine residues located within this segment must be maintained to permit full gene expression. This protein motif may describe boundaries for a STAT6 activation domain of the IL-4Rα that is responsible for initiation of IL-4 biological function within target tissues.

Initial characterization of the IL-4R demonstrated that all cell types express the IL-4Rα chain. However, the γ_c is only expressed on hemopoietic cells, raising the question of how nonhemopoietic cells induce IL-4 signaling. There is evidence that the γ_c is not required for IL-4 signaling. After treatment with IL-4, THP-1 cells, COS-7 cells, and Mono Mac 6 cells, all of which lack γ_c, are capable of activating STAT6 (20, 28–30). In addition, other IL-4-responsive cell lines, such as the plasmacytoma B9 and renal cell lines, do not express the γ_c (20, 31). B cells derived from patients with X-linked SCID, a disease characterized by disruption of the γ_c gene, do not respond to IL-2, yet maintain their ability to respond to IL-4, as measured by proliferation, IgE synthesis, and increased surface expression of IgM and CD23 (32). These data demonstrate that γ_c is not necessary for IL-4R signaling and suggest that IL-4Rα can signal by ligand-induced homodimerization, or, perhaps, can interact with another signaling chain.

To determine whether the IL-4Rα chain can independently activate STAT6 and induce IL-4-specific responses, a chimeric receptor was constructed containing the transmembrane and intracellular regions of the IL-4Rα chain and the extracellular region of c-*kit*, a receptor tyrosine kinase that homodimerizes upon binding stem cell factor (SCF). Multiple IL-4-specific functions were induced in response to SCF in lymphocytes expressing the chimeric receptor chain, thereby defining a new model in which IL-4R signaling can occur in the absence of γ_c and JAK-3.

Materials and Methods

Cell lines and culture conditions

M12.4.1 is an Ig-negative, class II^+, HGPRT-deficient variant of M12.4 that has been previously described (33) and was originally derived from the M12 B lymphoma. M12.4.1 cells were cultured at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO), 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. CTLL2 is an IL-2-dependent, IL-4-responsive T cell line that was maintained as previously described (34). The 1D4 cell line is derived from the HeLa line, and both the mutant JAK-1-deficient 1D4 and the parental 1D4 line were generously provided by Dr. Richard Flavell (35).

Generation of cell lines expressing the chimeric receptor

Both the murine c-*kit* cDNA and human IL-4Rα cDNA were gifts from Immunex (Seattle, WA). The kit/IL-4Rα chimeric construct was generated by a two-step PCR using the fusion primers CAA ATC CAG GCC CAC *CTC CTG CTG GGC GTC* and *GAC GCC CAG CAG GAG* GTG GGC CTG GAT TTG, in which the italicized bases are complementary to IL-4Rα, and the remainder are complementary to c-*kit* (36).

Transfections of M12.4.1 were performed by electroporation using CellZapII (Anderson Electronics, Brookline, MA). Briefly, 20 μg of CsCl-purified plasmid DNA was pipetted into a sterile electroporation cuvette. A total of 20×10^6 cells were resuspended in 0.4 ml RPMI 1640 and then added to the cuvette. After mixing well, the cells were electroporated at 1050 μF/240 V, pipetted into complete medium at $\sim 1 \times 10^6$ cells/ml, and plated in 24-well plates. G418 at a final concentration of 1 mg/ml was added at 24 h, and colonies were then grown exclusively in selection medium. Transfection and selection of CTLL2 cells were performed as previously described (34).

Cytokines

SCF was a gift from Immunex and used at a concentration of 40 ng/ml. Murine IL-4 was a gift from Dr. Robert Coffman of DNAX Research Institute (Palo Alto, CA) and was used at a concentration of 50 ng/ml. Human rIL-4 was a gift from Dr. Satwant Narula (Schering-Plough Corp., Kenilworth, NJ) and was used at a final concentration of 100 U/ml. Human rIL-2 was a gift from Dr. Ned Braunstein.

Antibodies

Rat anti-mouse c-*kit* (ACK-2; Life Technologies, Grand Island, NY) at a concentration of 0.3 μg/100 μl and rat anti-human IL-4Rα (clones M8 and M10) at a concentration of 1.0 μg/100 μl (Immunex Corp.) were secondarily labeled with FITC-conjugated goat anti-rat (Life Technologies) at a concentration of 0.5 μg/100 μl. FITC-conjugated rat anti-mouse CD23 (clone B3B4; PharMingen, San Diego, CA) was used at a final concentration of 0.5 μg/100 μl. Rabbit anti-human/mouse JAK-1, rabbit anti-mouse JAK-3, and anti-phosphotyrosine (clone 4G10) were used at a dilution of 1/1000 (Upstate Biotechnology, Inc., Lake Placid, NY). Polyclonal anti-STAT6-specific Abs were generated by immunizing a rabbit with a peptide corresponding to amino acids 805 to 823 of murine STAT6 (Santa Cruz Biotechnology, Santa Cruz, CA).

FACS analysis

Transfected and parental cells were incubated with the designated cytokine for 48 h at 37°C. Cells were collected and washed three times in ice-cold PBS before resuspension in 3% BSA/0.02% sodium azide in PBS at a concentration of 1×10^6/ml. One hundred microliters of cell suspension were incubated with Ab in the appropriate dilution at 4°C for 30 min. Cells were washed and stained with a conjugated secondary Ab. Cells were washed again three times; resuspended in PBS containing 3% BSA. 0.02% sodium azide, and 100 μg/ml propidium iodide for dead cell exclusion; and analyzed on a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA).

Proliferation assay

Standard [^{3}H]TdR incorporation assays were performed as previously described (34).

Preparation of cell extracts

Whole cell extracts were prepared for electrophoretic mobility shift assay (EMSA), immunoprecipitation (IP), and in vitro kinase assay as previously described (37).

Oligonucleotide probe for EMSA

A double-stranded oligonucleotide probe from the interferon response factor 1 (IRF-1) GAS element spans from −138 to −107 of the IRF-1 promoter TACAACAGCCTGA**TTTCCCCGAA**TGACGGC and includes the IRF-1 GAS (shown in bold). Double-stranded oligonucleotides were 3′ end labeled with Klenow enzyme and [α-^{32}P]dCTP (3000 Ci/mmol).

EMSA

DNA binding reactions were performed as previously described (8) in 10-μl reaction volumes containing 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 20 mM HEPES (pH 7.9), 4% Ficoll, 1.2 mg/ml BSA, 200 μg/ml poly(dI-dC:dI-dC), 5 μg of extract, and 1 ng of probe. After a 20-min incubation at room temperature, binding reactions were fractionated on 0.22× Tris-buffered EDTA/4.5% acrylamide (29/1) gels. Supershift experiments were performed by adding 0.5 μl of antiserum in a 10-μl binding reaction volume and incubating at 4°C for 30 min.

Immunoprecipitation and Western blotting

Whole cell extracts were precleared by the addition of 3 μl of preimmune serum with protein A-Sepharose (Oncogene Sciences, Manhasset, NY) beads per 300 to 1200 μl of extract. After mixing at 4°C for 2 h, supernatants were collected and divided into 600-μl aliquots. Antiserum (1/100 dilution) was added to each of these samples and allowed to incubate at 4°C overnight. Subsequently, 15 μl of protein A-Sepharose was added, and samples were agitated for an additional 90 min. The IP were then collected and washed three times in 500 μl of lysis buffer. The pellet was resuspended in 40 μl of SDS gel loading buffer, boiled for 2 min, and separated on 7% PAGE. Proteins were transferred to a nitrocellulose membrane using a semidry blotter (Bio-Rad Transblot, Bio-Rad Laboratories, Richmond, CA) for 30 min at 18 V and then subjected to immunoblotting as previously described (38).

In vitro kinase assay

After IP for either JAK-1 or JAK-3, samples were washed three times in lysis buffer, washed twice in kinase buffer (10 mM HEPES, pH 7.4; 2 mM MnCl; 10 mM $MgCl_2$; 1 mM DTT; 0.1 mM Na_3VO_4; 150 mM NaCl; and

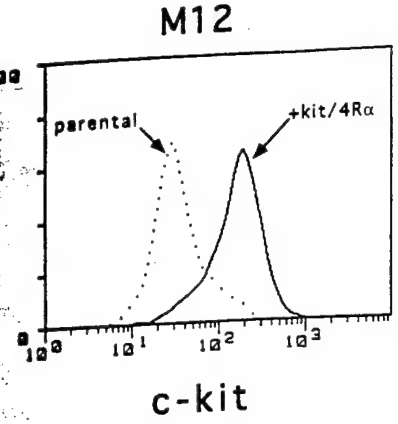

FIGURE 1. Expression of the chimeric kit/IL-4Rα chain on the surface of M12.4.1 cells. Cells were stably transfected with an expression vector encoding the chimeric receptor and selected for resistance to G418. Viable colonies were then screened for cell surface expression of the extracellular domain of c-*kit* with the rat Ab ACK-2 and FITC-conjugated goat anti-rat secondary Ab. All subsequent experiments were performed with cells derived from a single subclone and maintained on G418. Similar expression levels were obtained for CTLL2 transfectants (data not shown).

1 mM PMSF), and resuspended in 40 μl of kinase buffer. At room temperature, 3 μl of [γ-^{32}P]ATP (3000 Ci/mM) was added, and the reaction was allowed to run for 30 min. Samples were washed four times in kinase buffer, separated by SDS-PAGE, and visualized by autoradiography.

Isolation of RNA and RNA blot analysis

Both M12.4.1 parental cells and M12-kit/IL-4Rα transfectants were either not treated or treated with IL-4 or SCF for 48 h. Total cell RNA was prepared by the guanidinium isothiocyanate-CsCl method, and RNA blots were prepared and hybridized as previously described (39). The Cε117 probe is a 1-kb *Eco*RI cDNA fragment that recognizes a 1.7-kb Cε-hybridizing band. The blot was stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

Results

The chimeric receptor kit/IL-4Rα induces activation of STAT6 in response to SCF

The cytoplasmic domain of the IL-4Rα chain is critical for activation of STAT6 (27). To assess whether it might also be sufficient, we sought to create an experimental system that would activate the IL-4Rα chain independently of γ_c or other potential receptor chains. A c-kit/IL-4Rα chimeric receptor offered several advantages; namely, 1) SCF is a homodimeric ligand that exclusively binds and induces homodimerization of the c-*kit* chain (40); 2) chimeric c-*kit*/IL-2R and c-*kit*/erythropoietin receptor chains have previously been shown to generate IL-2- and erythropoietin-like signals, respectively, in response to SCF (34, 41); and 3) wild-type c-*kit* is unable to activate JAK-1, JAK-3, STAT6, or other components of the IL-4R signaling cascade (16). A chimeric receptor chain was constructed containing a precise fusion between the transmembrane and intracellular regions of the human IL-4Rα chain and the extracellular domain of murine c-*kit* (kit/IL-4Rα). The B cell line M12.4.1 and the T cell line CTLL2 were transfected with an expression vector encoding kit/IL-4Rα and selected for resistance to G418. Subclones were screened for cell surface expression of kit/IL-4Rα by flow cytometry with ACK-2, an Ab specific for murine c-*kit* (Fig. 1).

To evaluate the ability of cells expressing kit/IL-4Rα to activate STAT6, EMSAs were performed using an oligonucleotide probe that was initially characterized as GAS, but binds several STATs, including STAT6 (8, 16). Treatment of either M12-kit/IL-4Rα or CTLL2-kit/IL-4Rα transfectants with SCF resulted in activation of a GAS-binding protein complex with the same mobility as STAT6, which was induced by stimulation with IL-4 (Fig. 2, *A* and *B*). Antiserum specific for STAT6 supershifted both the SCF- and IL-4-induced complexes. To confirm that this response required the cytoplasmic domain of IL-4Rα, transfectants expressing a chimeric kit/IL-2Rβ chain were also generated. Treatment of M12-kit/IL-2Rβ with SCF induced activation of a complex with faster mobility then STAT6. Experiments with STAT5 antisera identified this complex as STAT5 (data not shown). Treatment of parental M12 cells with SCF failed to induce GAS binding activity. These data demonstrate that the cytoplasmic region of the IL-4Rα chain contains all the signaling domains necessary for the activation of STAT6.

The chimeric receptor kit/IL-4Rα induces tyrosine phosphorylation of JAK-1, but not JAK-3

The current model for IL-4 signaling presumes that ligand-induced heterodimerization of IL-4Rα and γ_c leads to activation of JAK-1 and JAK-3. In cells expressing the kit/IL-4Rα receptor, oligomerization of this chimeric receptor by SCF should only activate the IL-4Rα-associated JAK-1 kinase. Alternatively, the lymphocyte cell lines containing this chimeric receptor express endogenous γ_c and JAK-3. Therefore, it is possible that these molecules are recruited into the receptor complexes via interactions with either the transmembrane or cytoplasmic domains of the IL-4Rα component of the chimeric receptor molecules. To further define the roles of these kinases in IL-4 signaling, cells expressing the chimeric receptor were examined for JAK kinase activation.

Lysates from cells treated with no cytokine or with IL-4 or SCF were immunoprecipitated with Abs against JAK-1 or JAK-3 and probed with an Ab to phosphotyrosine (Fig. 3*A*). Tyrosine phosphorylation of JAK-1 was strongly induced by SCF in cells expressing kit/IL-4Rα, whereas the magnitude of JAK-3 phosphorylation was unaffected (Fig. 3*A*). To determine whether tyrosine phosphorylation of JAK-1 correlated with increased catalytic activity, in vitro kinase assays were performed. M12-kit/IL-4Rα cells treated with SCF showed increased JAK-1 kinase activity, comparable in magnitude to that immunoprecipitated from IL-4-treated cells (Fig. 3*B*). As expected, the catalytic activity of JAK-3 is not affected by SCF treatment, but is markedly increased in response to IL-4. Thus, SCF binding to the chimeric kit/IL-4Rα-chain leads to the activation of JAK-1, but not JAK-3. Therefore, in these cells JAK-3 catalytic activity is not required for induction of STAT6 homodimerization.

Activation of STAT6 by the IL-4R requires JAK-1

The kinase data suggest that the activation of JAK-1 by kit/IL-4Rα is sufficient for induction of STAT6 DNA binding activity. To determine whether JAK-1 is essential for STAT6 activation, we examined the ability of JAK-1-deficient cells to activate STAT6 in response to IL-4. Mutant HeLa cells unresponsive to IFN-γ were previously generated using a selection system in which the herpes virus thymidine kinases gene was placed under the control of the HLA-DRα regulatory sequence, and colonies were selected for resistance to IFN-γ and gancyclovir (35). One of the classes of mutants generated was characterized by the complete loss of all IFN-γ responses and the loss of IFN-α-specific gene activation. Subsequent analysis demonstrated that defective signaling within these cells was due to a loss of JAK-1 expression.

To examine the role of JAK-1 in IL-4 signaling, extracts from parental and JAK-1-deficient cells were treated with IL-4 and examined for activation of STAT6 by EMSA. In the parental HeLa

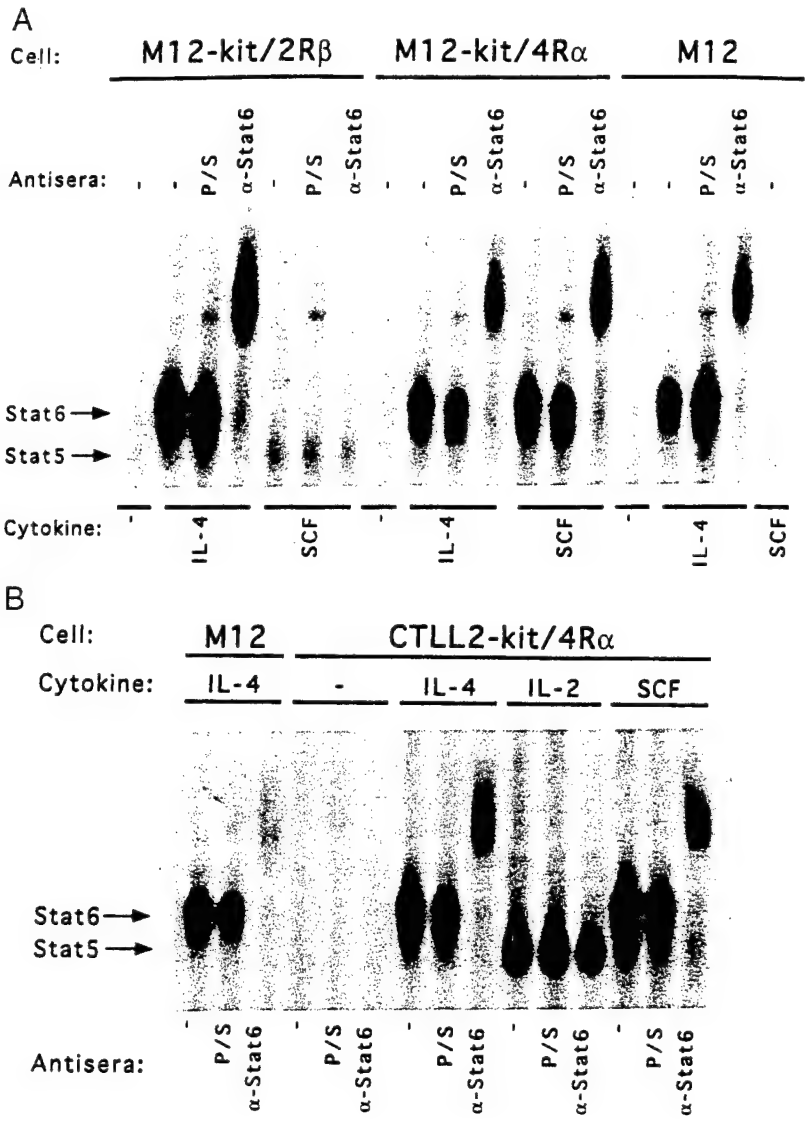

FIGURE 2. STAT6 DNA binding is induced by IL-4 or SCF. CTTL2 cells and M12.4.1 cells expressing kit/IL-4Rα were assayed for GAS-binding protein complexes using gel retardation assays. CTLL2 cells were deprived of IL-2 for 4 h before lysis and extraction of nuclear proteins. Extracts were derived from cells stimulated for 30 min with medium alone, IL-4, or SCF. The presence of STAT6 in each complex was assessed by supershift analysis using preimmune serum as a negative control or the STAT6-specific antiserum as indicated.

cell line, STAT6 is activated in response to IL-4, and a STAT6-specific Ab blocks GAS binding activity (Fig. 4). In contrast, STAT6 is not activated in response to IL-4 in the JAK-1-deficient cell line. These data identify a critical role for JAK-1 in the activation of STAT6 by IL-4.

The chimeric receptor kit/IL-4Rα induces T cell proliferation in response to SCF

IL-4 is a known mediator of lymphocyte proliferation. Within the cytoplasmic domain of IL-4Rα, the insulin receptor, and the insulin-like growth factor receptor is a highly homologous region denoted the I4R motif, which contains a central tyrosine (Y497) critical for the tyrosine phosphorylation of insulin receptor substrate (IRS-1) and induction of cell proliferation in response to IL-4 (42). To determine whether homodimerization of the IL-4Rα cytoplasmic domain and activation of JAK-1 can induce T cell proliferation, CTLL2 cells expressing kit/IL-4Rα were assayed for incorporation of [^{3}H]thymidine after stimulation with IL-2, IL-4, or SCF. In two independent CTLL2-kit/IL-4Rα subclones, ligation of kit/IL-4Rα induced a proliferative response on the same order of magnitude as that mediated by the endogenous IL-4R, which was about fourfold less than the response to IL-2 (Fig. 5). This indicates that the IL-4Rα chain can induce T cell proliferation independent of γ_c and JAK-3.

The chimeric receptor kit/IL-4Rα induces CD23 surface expression and transcription of the germ-line ϵ locus in response to SCF

Treatment of cells expressing kit/IL-4Rα with SCF resulted in the activation of STAT6. We next sought to determine whether this was sufficient to induce transcription of IL-4-responsive genes. A STAT6 binding site has been identified in the IL-4-responsive CD23 promoter. Confirmation of the importance of STAT6 in the induction of CD23 is provided by STAT6-deficient mice, which are unable to induce CD23 in response to IL-4. Parental M12.4.1 cells demonstrate increased surface expression of CD23 in response to IL-4, but not SCF (Fig. 6). In contrast, treatment of transfectants expressing kit/IL-4Rα with either IL-4 or SCF results in equivalent levels of expression of CD23. Thus, kit/IL-4Rα contains all the signaling domains necessary to induce CD23 expression in lymphocytes.

In mice, IL-4 is essential for Ig heavy chain class switching to IgE (43). The targeting of class switching to IgE by IL-4 is mediated by the induction of germ-line Cϵ transcripts (44). An essential STAT6 binding site has been identified within the promoter of the germ-line Cϵ gene (21). To determine whether signal transduction via the IL-4Rα chain is sufficient for the induction of germ-line ϵ transcripts, M12.4.1 cells were treated with medium

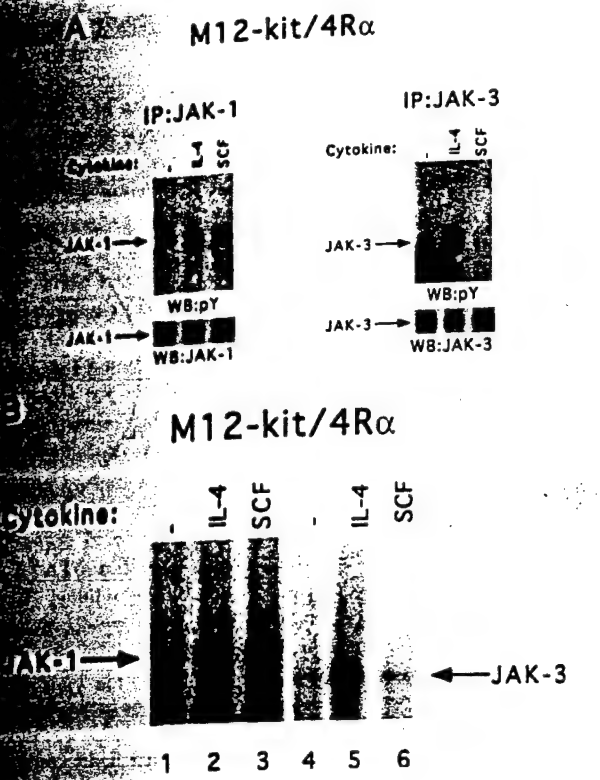

FIGURE 3. Activation of JAK-1, but not JAK-3, by kit/IL-4Rα. *A*, M12.4.1-kit/IL-4Rα transfectants were treated with medium alone, IL-4, or SCF at 37°C for 15 min. Proteins were immunoprecipitated with either JAK-1 or JAK-3 Abs, fractionated by SDS-PAGE (7% gel), and blotted with Abs to phosphotyrosine. The blots were then stripped and reprobed with Abs to either JAK-1 or JAK-3, as indicated, to confirm equivalent loading of protein across lanes. *B*, For in vitro kinase assays, immunoprecipitated proteins were resuspended in kinase buffer and incubated with [γ-^{32}P]ATP at room temperature for 30 min. Complexes were fractionated by SDS-PAGE and visualized by autoradiography.

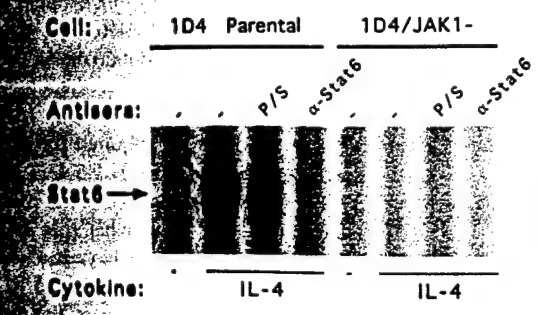

FIGURE 4. JAK-1 is required for activation of STAT6. Parental 1D4 cells or a mutant derivative of 1D4 that lacks JAK-1 were treated with either medium alone or IL-4 for 15 min, and whole cell extracts were analyzed by EMSA. STAT-6-specific antiserum was used for supershift of complexes.

alone, IL-4, or SCF and analyzed by Northern blotting for expression of germ-line ε transcripts (Fig. 7). Transfected cells expressing kit/IL-4Rα synthesized germ-line ε transcripts in response to either IL-4 or SCF, whereas the parental cell line was responsive to IL-4 only. Thus, kit/IL-4Rα is competent to induce germ-line ε transcripts in response to SCF, indicating that the cytoplasmic domain of the IL-4Rα chain can independently mediate signals regulating the expression of IL-4-specific genes.

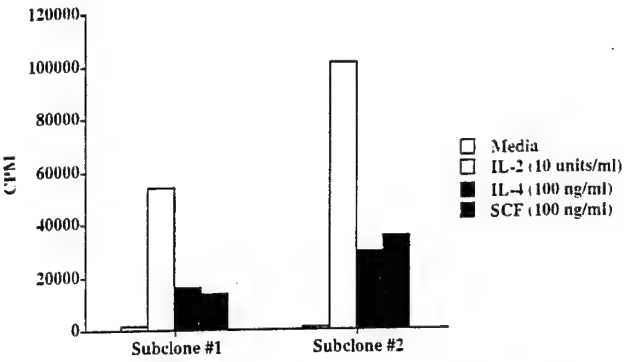

FIGURE 5. Homodimerization of the IL-4Rα cytoplasmic domain is sufficient for T cell proliferation. CTLL2 cells transfected with kit/IL-4Rα were stimulated overnight with medium alone, IL-2, IL-4, or SCF, and proliferation assays were performed as previously described. Results are shown for two independent transfected subclones.

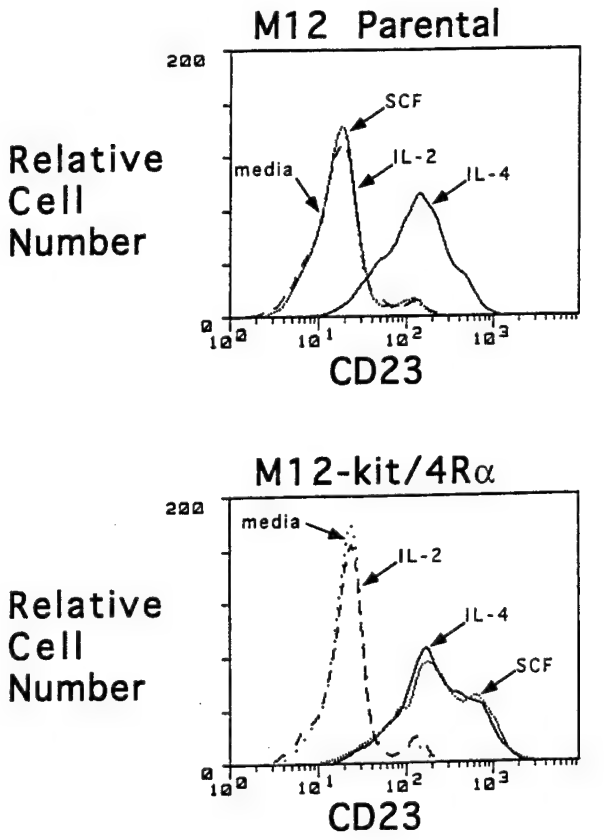

FIGURE 6. Cell surface expression of CD23 is mediated by kit/IL-4Rα. Parental M12.4.1 cells or a subclone expressing c-kit/IL-4Rα were grown for 48 h in the presence of medium alone, IL-2, IL-4, or SCF. Cells were collected and immunostained with FITC-conjugated anti-murine CD23.

Discussion

Previous studies have indicated that the IL-4R consists of two subunits, IL-4Rα and γ_c. In transfection assays, cells expressing both IL-4Rα and γ_c had a higher affinity for IL-4 than those expressing IL-4Rα alone (5). In addition, a γ_c-specific Ab can inhibit IL-4-induced proliferation and high affinity binding of IL-4 to

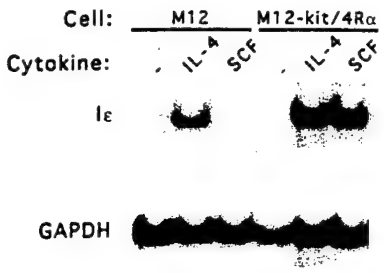

FIGURE 7. Synthesis of germ-line ε transcripts is induced by kit/IL-4Rα in response to SCF. Parental M12.4.1 cells or transfectants expressing kit/IL-4Rα were grown in the presence of no cytokine or with IL-4 or SCF for 48 h. RNA was extracted and separated on a standard formaldehyde gel, blotted to Nytran Plus (Schleicher and Schuell, Keene, NH), probed with radiolabeled Cε cDNA, and then stripped and reprobed for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts.

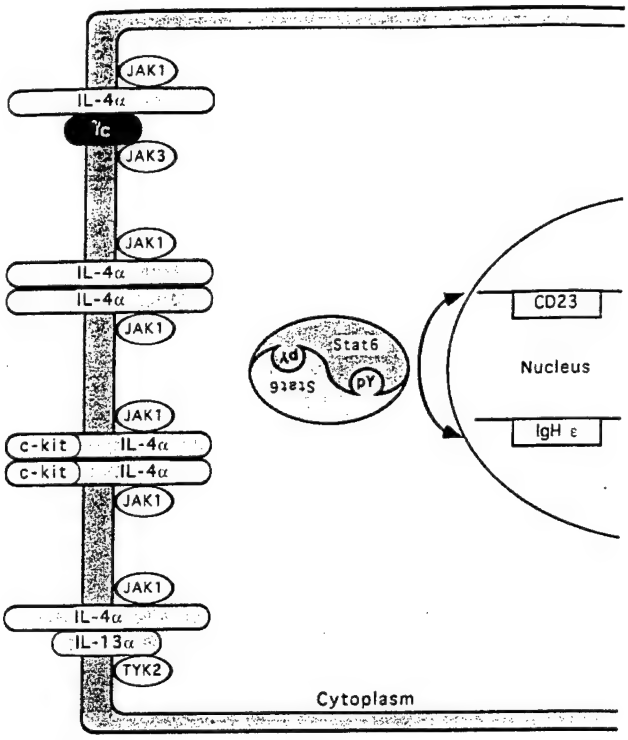

FIGURE 8. Model for signaling by three distinct IL-4R complexes. The three different IL-4R proposed are composed of IL-4Rα/γ_c heterodimers, IL-4Rα/IL-4Rα homodimers, or IL-4Rα/IL-13Rα heterodimers. All three receptor complexes use IL-4Rα and JAK-1 and can activate STAT6 by tyrosine phosphorylation (pY), leading to the transcription of IL-4-specific genes. The chimeric receptor kit/IL-4Rα is shown spanning the cell membrane. Two IL-4-specific target genes that require STAT6 are shown in the nucleus, namely CD23 and Iε.

CTLL2 cells (10). Furthermore, in cells transfected with full-length IL-4Rα and cytoplasmic truncation mutants of γ_c, responses to IL-4 were inhibited, as measured by *fos*-chloramphenicol acetyltransferase activity and proliferation assays, compared with cells in which full-length γ_c was cotransfected with IL-4Rα (45). Despite these in vitro data, however, lymphocytes derived from X-chromosome-linked SCID patients, who have a disrupted γ_c gene, are able to respond to IL-4 (32). In addition, JAK-3 knockout mice can activate STAT6 (46). Therefore, an alternative IL-4R complex must form in the absence of γ_c.

These results suggested two paradigms for IL-4 signaling: one involving heterodimerization of IL-4Rα and γ_c, and another in which IL-4Rα interacts with a surrogate γ′-chain. A candidate for the γ′-chain is the recently cloned IL-13Rα chain, which interacts with the IL-4Rα chain in the presence of IL-13 (47). Alternatively, because IL-4Rα is able to bind IL-4 with picomolar affinity in the absence of γ_c, we hypothesized that this chain might signal by ligand-induced homodimerization in cells lacking γ_c. A chimeric receptor chain, kit/IL-4Rα, induced multiple signaling events characteristic of the IL-4 response, including activation of JAK-1 and STAT6, expression of CD23 and germ-line ε transcripts, and cellular proliferation. Of particular significance, the kinase JAK-3, which associates with and is activated by the γ_c, was not activated by SCF, indicating that endogenous γ_c and JAK-3 are not recruited into the chimeric receptor complex. Rather, signaling appears to be mediated by the IL-4Rα-associated kinase JAK-1. These results provide an alternative mechanism for IL-4R signaling in cell types lacking γ_c and IL-13Rα and demonstrate that a surrogate γ′-chain is not necessarily required to induce IL-4-specific responses.

Previous studies suggest that chimeric receptors containing the extracellular domain of c-*kit* can be homodimerized by SCF. SCF is a noncovalent homodimeric glycoprotein and normally is only able to bind the dimeric form of c-*kit* (40). Studies using chimeric IL-2R subunits composed of the extracellular domain of c-*kit* show indistinguishable patterns of IL-2-induced signaling compared with cross-linking Abs (34, 41). Furthermore, alternative chimeric IL-2R chains composed of the extracellular regions of the α- and β-chains of the heterodimeric granulocyte-macrophage CSF receptor functioned similarly.

Therefore, we propose that there may, in fact, be three types of IL-4R: namely, one characterized by heterodimerization of IL-4Rα and γ_c, a second composed of the IL-4Rα and the recently cloned IL-13Rα, and a third in which signaling is mediated by homodimerization of IL-4Rα (Fig. 8). Whether these three putative receptor complexes induce the same or different biological responses remains to be determined. However, at a biochemical level these signals differ, as heterodimerization of IL-4Rα and γ_c activates both JAK-1 and JAK-3, whereas homodimerization of IL-4Rα chain activates JAK-1 only.

The results with kit/IL-4Rα together with data from JAK-3-deficient mice demonstrate that JAK-3 is not required for activation of STAT6 by the IL-4R. In contrast, JAK-1-deficient HeLa cells failed to activate STAT6, demonstrating an essential role for this kinase in IL-4R signaling. It is not clear what role JAK-3 might play in IL-4R signaling. To date, no genes have been identified that are differentially transcribed in response to IL-4 in the presence or the absence of γ_c. Theoretically, however, the differential expression of γ_c on hemopoietic vs nonhemopoietic cells may impart tissue specificity to IL-4-mediated gene expression.

An additional possibility is that γ_c serves a mitogenic function in the IL-4R. While we have shown that the cytoplasmic domain of the IL-4Rα chain is sufficient for the activation of JAK-1 and can mediate a proliferative signal in CTLL2 cells as measured by 24-h [^{3}H]thymidine incorporation, long term growth of the IL-4-responsive T cell clone D10 could not be supported by ligation of kit/IL-4Rα (B. H. Nelson, unpublished observations). This suggests that γ_c might be required for full mitogenic signaling by the IL-4R in lymphocytes, much as the chain is required for IL-2-induced proliferative responses in T cells.

Other studies using chimeric receptor chains have shown that the IL-4Rα cytoplasmic domain can mediate biochemical events

characteristic of the IL-4R signal. In one study, the cytoplasmic domains of human IL-4Rα and γ_c were exchanged, and the two resulting constructs were cotransfected into murine Ba/F3 cells. Upon binding of an Ab specific to an extracellular epitope tag on the extracellular γ_c/intracellular IL-4Rα chimeric chain, STAT6 and JAK-1 were activated, suggesting that the cytoplasmic domain of γ_c and the associated JAK-3 molecule were dispensable for these aspects of signaling (48). In a second study involving a construct composed of the IL-4Rα chain cytoplasmic domain and the erythropoietin receptor extracellular region, JAK-1, STAT6, and insulin receptor substrate 1 (IRS-1) were activated upon treatment with erythropoietin (49). Our study extends these findings by showing that homodimerization of the cytoplasmic domain of IL-4Rα induces not only activation of JAK-1 and STAT6, but also T cell proliferation and transcription of endogenous IL-4-specific genes. The results thus provide in vivo evidence that a functional signal can be transmitted by IL-4Rα homodimerization.

We have characterized a unique mechanism of IL-4R signal transduction in which homodimerization of IL-4Rα chains activates STAT6, induces the transcription of IL-4 target genes, and mediates cellular proliferation. IL-4 is a key cytokine in the generation of allergic responses. Because IL-4 signaling can occur without activation of γ_c and JAK-3, inhibiting the activity of these proteins may not be sufficient for altering allergic responses. In contrast, IL-4Rα, JAK-1, and STAT6 may be appropriate targets for blocking the transcription of IL-4-specific genes and, therefore, enabling modulation of allergic diseases.

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IL-4 Receptor Mutations

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Abbreviations

IL	Interleukin
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
4PS	IL-4 Phosphorylation Substrate
IRS	Insulin Receptor Substrate
I4R	Insulin IL-4 Receptor
Dok	Downstream of kinase
FRIP	IL-4 Receptor Interacting Protein
SH2	Src-homology-domain 2
STAT	Signal Transducer and Activator of Transcription
SHP	SH2-containing tyrosine phosphatase
SHIP	SH2-containing inositol phosphatase
SOCS	Suppressor of Cytokine Signaling

Summary

Interleukin-4 plays an important role in regulating immune responses. Distinct signaling pathways, including gene activation/differentiation and proliferation/protection from apoptosis, are initiated from the IL-4 receptor complex upon ligand-receptor engagement. Several advances have been made in our understanding of how distinct functions of IL-4 are mediated. Most of these studies employed artificial mutations of IL-4 receptor α chain using site-directed mutagenesis and/or deletional mutation. In addition, naturally occurring mutations of IL-4Rα have been identified and implicated as a genetic predisposition for allergic disorders. The results of these studies suggest a modular organization of the receptor and an independent regulation of gene activation and cell growth.

Introduction

Interleukin-4 is a pleiotropic and multifunctional cytokine produced by activated T cells, mast cells, and basophils (1). In addition, a specialized subset of T cells ($NK1.1^+$) and eosinophils have also been reported to produce IL-4 (2, 3). IL-4 plays a critical role in regulating the outcome of an immune response by facilitating type 2 T helper cell differentiation and suppressing the differentiation of IFNγ-producing $CD4^+$ T cells, thereby favoring humoral immune responses (4, 5). The other important function of IL-4 is the regulation of immunoglobulin class switching. It induces class switching to IgE and IgG4 in human B cells and in mouse B cells to IgE and IgG1(6-8). The increased levels of IgE reactive to allergens associated with atopic diseases suggest a preeminent role in the regulation of such conditions for IL-4.

In addition to its direct involvement in regulating immune responses, IL-4 also exerts a wide variety of effects on hematopoietic and non-hemopoietic cells. It enhances the expression of CD23 and class II MHC molecules in B cells, and up-regulates surface expression of the IL-4 receptor (9-11). On vascular endothelial cells, IL-4 together with TNF induces the expression of vascular cell adhesion molecule-1 and down-regulates the expression of E-selectin, thereby changing the adhesive characteristics of endothelial cells (12, 13). The altered adhesive characteristics of endothelial cells facilitate tissue infiltration of allergic inflammatory cells, such as eosinophils.

IL-4 receptors are expressed in hematopoietic cells and a range of non-hematopoietic cells including epithelial, endothelial, muscle, fibroblast, and liver cells (14, 15). In

hematopoietic cells, the IL-4 receptor complex is composed of a 140-kDa high affinity ligand-binding chain, IL-4 receptor α chain (IL-4Rα) and common γ chain (γC) shared by IL-2, IL-7, IL-9, and IL-15 (1). In contrast, the IL-13Rα' is the predominant accessory chain of the IL-4 receptor complex in non-hematopoietic cells (16). Furthermore, the IL-4Rα chain together with the IL-13Rα and IL-13Rα' constitutes the IL-13 receptor complex (17-19). This may explain the redundancy in biological responses mediated by IL-4 and IL-13. Both IL-4 and IL-13 have been implicated in allergic diseases, probably through redundant and independent pathways (20-22).

Although homodimerized IL-4Rα can generate biological signals within the cell, physiologic signaling requires heterodimerization of IL-4Rα and the accessory chain. Neither IL-4Rα nor γC, both belonging to the hematopoietin receptor superfamily, contains intrinsic kinase activities. However, upon IL-4 stimulation tyrosine kinases are activated, resulting in the phosphorylation of cellular substrates and the initiation of signaling cascades. Three members of the Janus kinase family, Jak-1, Jak-2, and Jak–3, have been shown to be activated and to associate with the components of the IL-4 receptor complex (23). Jak1 has been proposed to bind the IL-4Rα whereas Jak-3 associates with the γC chain (24-26). Jak-2 has been shown to associate with the IL-4Rα in some cell lines (27).

Upon ligand-receptor engagement, the IL-4Rα chain itself is immediately tyrosine-phosphorylated. Five conserved tyrosine residues (Y497/Y1, Y575/Y2, Y603/Y3, Y631/Y4, and Y713/Y5) that can potentially be phosphorylated are present in the

cytoplasmic domain of IL-4Rα(1). After tyrosine phosphorylation, these conserved tyrosine residues become potential docking sites for downstream signaling molecules containing, Src-homology 2 (SH2) or phosphotyrosine-binding (PTB) domains. It remains unclear as to which tyrosine kinase actually phosphorylates the IL-4 receptor and the substrates that are recruited to the receptor.

Studies employed mutagenesis of the IL-4Rα chain have generated informative results that let to the biochemical characterization of independent signaling pathways initiated from distinct domains of the IL-4Rα chain. The intracellular region of the IL-4Rα can be divided into four functionally distinct domains. The membrane proximal domain serves as an interaction site for the Janus kinase. The second domain, named the Insulin IL-4 Receptor (I4R) motif, contains the Y497 (Y1) residue and is critical in transmitting proliferative and anti-apoptotic signals. IL-4-induced gene activation has been attributed to the third domain containing three redundant tyrosine residues (Y2, Y3, and Y4) that, after tyrosine phosphorylation, serve as docking sites for the signal transducer and activator of transcription 6 (Stat6). The last domain contains the Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) whose function remains unclear but has been implicated in the down-modulation of proliferative signals induced by IL-4. Here we summarize our current understanding of the signaling mechanisms of the IL-4 receptor with a special emphasis on those results generated from the studies of the mutations, artificially generated or naturally occurring, in the human IL-4Rα chains. The mutagenesis approach has been informative because of the non-cossreactivity between human and murine IL-4.

The I4R motif

When the patterns of protein phosphorylation in response to IL-4 and IL-3 were compared, a unique 170-kDa phosphoprotein was identified in hematopoietic cell lines (28). This protein was initially named the IL-4 phosphorylation substrate (4PS) and later renamed as Insulin Receptor Substrate-2 (IRS-2) because of a high degree of homology to IRS-1 that expresses in cells of non-hematopoietic origin (29-31). The critical role of IRS-1/2 for the proliferation induced by IL-4 was demonstrated in the factor-dependent myeloid progenitor cell line 32D, which does not express detectable levels of IRS-1 or IRS-2. Whereas no IL-4-induced proliferation was observed in parental 32D cell line, other myeloid cell lines expressing IRS-2 and stable IRS-1 transfectants of 32D cells showed IL-4-dependent cell growth (30, 31).

The identification of IRS-1/2 binding motif within the cytoplasmic region of the IL-4Rα was accomplished by generating a series of truncation mutants of the human IL-4Rα chain. The deletional mutants indicated that the sequence between amino acid 437 and 557 of the IL-4Rα, in which the central Y497 resides, is important in the process of IRS recruitment/activation by IL-4 stimulation (32, 33). Indeed, a point mutant where Tyr 497 was changed into Phe greatly reduced the ability of the mutant receptor to signal IL-4-mediated proliferation and blocked IRS-1/2 activation, suggesting that once phosphorylated, Y497 is the docking site for IRS-1/2. The amino acid sequence surrounding Tyr 497 is ^{488}PL-$(x)_4$-NPxYxSSD^{502}, showing a high degree of homology to the sequences in the intracellular regions of insulin and IGF-1 receptors. Because this

motif is critical for the recruitment/activation of IRS-1/2 to the insulin receptor and the IL-4 receptor, it was termed the Insulin IL-4 Receptor (I4R) motif. I4R motif also plays a crucial role in the IL-4-mediated protection from apoptosis. The first indication of IRS-1/2's involvement in IL-4-mediated protection from apoptosis came from the observation that parental 32D cells lacking IRS-1/2 had less viable cells after IL-3 withdrawal when compared to stable IRS-1 transfectants of 32D (32D/IRS-1) cells (34). Further analysis revealed that human IL-4 was unable to prevent cell death in 32D/IRS-1 cells expressing the mutant human IL-4Rα chain in which the I4R motif was mutated at amino acid 497 (Tyr-to-Phe) (35). However, the anti-apoptotic signaling pathway downstream of IRS-1/2 remains undefined.

In addition to IRS-1/2, the I4R motif of the IL-4Rα chains can serve as a docking site for other signaling molecules. Recently, a homolog of the PTB-domain protein $p62^{dok}$ (Downstream of Kinases) named FRIP (IL-Four Receptor Interacting Protein) has been cloned by using the I4R motif from the IL-4Rα linked to the insulin receptor kinase as a bait in the yeast two-hybrid system (36). Both FRIP and $p62^{dok}$ are phosphorylated upon IL-4 stimulation and have been implicated in the negative regulation of the Ras/MAPK pathway for their ability to bind Ras GTPase activating protein (RasGAP) (36, 37). Mutation of the central tyrosine (Y497F) within the I4R motif of the IL-4Rα chain blocked IL-4-induced tyrosine phosphorylation of FRIP (1, 36, 37). Evidence supporting the role for FRIP in the down-regulation of Ras pathway came from the study of mice homozygous for the *hairless* allele (*hr/hr*), which is linked to *Frip* on mouse chromosome 14 (38). T cells from *hr/hr* mice express 3 to 5-fold lower levels of FRIP protein than

those isolated from the littermate control and exhibit a hyperproliferative phenotype when stimulated with anti-CD3 plus IL-2 or IL-4 (36). Moreover, overexpression of FRIP in 32D and A.E7 cells diminished MAPK activation and Ras/MAPK pathway-dependent gene activation, respectively, in response to IL-2 stimulation.

The gene activation domain of the IL-4Rα chain

While the critical sequence motif for the signaling pathway leading to IL-4 induced proliferation was mapped to the I4R motif, a separate domain within the intracellular tail of the IL-4Rα chain is essential for the induction of signaling pathways leading to gene activation (39). The murine B lymphoma cell line, M12.4.1, expressing a truncated human IL-4Rα chain terminated at residue 657 retained a full capacity in IL-4 mediated enhanced surface expression of CD23, IL-4Rα, and class II MHC molecules and germline Iε transcription when compared to cells expressing full-length receptor. However, truncation at amino acid 557 greatly diminished IL-4-induced activation of these genes (33, 40). This gene activation domain contains three conserved tyrosine residues (Y757, Y603, and Y631), once phosphorylated providing potential docking sites for SH2-containing proteins. Various combinations of point mutations in Y575, Y603, and Y631 were made to evaluate the relative contribution of the three tyrosine residues to IL-4-induced gene activation. These three tyrosine residues appeared to be redundant in that any one or any two could be mutated to phenylalanine without severely affecting the capacity of the human IL-4Rα to signal IL-4-induced gene expression. Mutations of all three tyrosine residues severely impeded the signaling pathways leading to the expression of IL-4-responsive genes.

The ITIM of the IL-4Rα chain

The last conserved tyrosine residue (Y713) whose function remains unclear is located in the very C-terminal domain of the IL-4Rα chain. The amino acid sequence flanking Y713 matches the canonical consensus (I/VxYxxL) of the Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM). The ITIM was first identified in the cytoplasmic domain of FcγRIIB and has been recognized in a growing family of inhibitory receptors (41, 42). Accumulating evidence suggests that the ITIM, when phosphorylated on tyrosine, becomes a docking site for SH2-containing tyrosine phosphatase-1 (SHP-1), SHP-2, and/or SH2-containing inositol 5-phosphatase (SHIP) (43-47). After recruitment to the inhibitory receptors, these phosphatases down-modulate the signaling cascades initiating from B cell receptors, FcγRII, FcεRI, and T cell receptors, presumably by dephosphorylating activated signaling molecules.

In vitro, a tyrosine-phosphorylated peptide corresponding to the putative human IL-4Rα (hIL-4Rα) ITIM but not its nonphosphorylated counterpart, co-precipitates SHP-1, SHP-2, and SHIP. Although *in vivo* all three SH2-containing phosphatases can associate with the IL-4 receptor complex, these SH2-containing phosphatases appear to be able to bind different motifs of IL-4Rα and various components of the IL-4 receptor complex. In an attempt to delineate the function of the putative IL-4Rα ITIM, mutant human IL-4Rα chains were made in which the ITIM was ablated by site-directed mutagenesis or by deletional mutation. Upon stimulation with human IL-4, both ITIM-deficient human IL-4Rα chains resulted in a hyperproliferative phenotype when expressed in the IRS-2 stable

transfectant of 32D myeloid progenitor cells, implicating a role for the IL-4Rα ITIM in the down-modulation of proliferative signals induced by IL-4 (P.-Y. Pan, C. Giallourakis, and P. B. Rothman, unpublished data). In contrast, no significant difference was observed in IL-4 mediated protection from apoptosis. Further analyses revealed that the ability of the ITIM mutants to mediate heightened proliferation correlated with an increased level of Stat6 tyrosine phosphorylation. Therefore, the IL-4Rα ITIM appears to be able to regulate IL-4 induced proliferation and Stat6 activation signaling pathways. However, whether the regulation of proliferative and gene activation signals by the IL-4Rα ITIM is independent of the IRS signaling pathway is yet to be determined.

The carboxyl-terminal domain containing Y713 has also been implicated in the IL-4-induced anti-apoptotic signaling pathway. Although mutation of Y713 to phenylalanine had no significant effect on the prevention from apoptosis induced by IL-4, transplantation of the domain between amino acid 666 to 768 to the truncated IL-2Rβ conferred protection from apoptosis (35). Interestingly, the Y713F mutation of the IL-2Rβ/IL-4Rα chimeric receptor drastically reduced the capacity of the receptor to signal IL-4-induced protection from apoptosis. The reason for the discrepancy between the results from the human IL-4Rα chain and those from the IL-2Rβ/IL-4Rα chimeric receptor remains unclear. One possibility is that in the context of the full length human IL-4Rα chain, the point mutation at Y713 could still allow partial protein binding. Alternatively, the anti-apoptotic signaling initiated from Y713 is redundant in the presence of I4R motif.

The naturally occurring mutations of IL-4Rα chain

Asthma and allergic disorders are complex genetic diseases with a number of contributing genes, which must interact with some environmental insult for the disease state to manifest. Among the genes implicated in the genetic predisposition of atopy are two naturally occurring variants of the IL-4Rα chain. The first novel IL-4Rα allele was identified in which guanine was substituted for adenine at nucleotide 1902 (48). This point mutation results in a mutation from glutamine to arginine at amino acid 576 (Q576R), the residue next to the first of the three redundant Stat6 binding sites, in the cytoplasmic tail of the IL-4Rα chain. Phosphopeptides derived from this domain of the mutant allele showed decreased binding of SHP-1 when compared to those derived from wild-type allele. The decreased binding of SHP-1 to the IL-4Rα in the presence of the Q576R mutation suggests prolonged activation/phosphorylation of signaling molecules. Indeed, IL-4 induced higher levels of CD23 expression in peripheral-blood mononuclear cells isolated from R576 homozygous patients than from wild-type homozygotes. However, the observed phenotype in B cells isolated from homozygous patients could not be recapitulated in the studies using murine lymphoma cell line M12.4.1 or myeloid progenitor cell line 32D expressing the mutant Q576R human IL-4Rα chain. The Q576R mutation did not lead to a significant change in the signaling capacity (Stat6 phosphorylation/DNA binding activity, CD23 induction, proliferation and protection from apoptosis) of the receptor in the context of two murine cell lines (49, 50). It is possible that additional alterations in the IL-4Rα chain or/and mutations in the signaling molecules of the IL-4 receptor complex, such as Janus kinases, Stat6, or SH2-containing phosphatases, are necessary for the effect of the Q576R mutation to be observed. Further

biochemical and functional analyses of the IL-4 signaling pathway in B cells isolated from Q576R homozygous patients are required to delineate the mechanisms.

The second IL-4Rα variant identified from allergic patients is an extracellular variant in which Val50 is substituted by Ile (51). Peripheral-blood mononuclear cells isolate from Ile50 bearing individuals showed enhanced CD23 induction and IgE production in response to IL-4. In contrast to a murine extracellular variant that has been shown to alter the ligand-binding affinity, no difference in binding assays was detected between Val50 and Ile50 alleles. Significantly, human and mouse B cell lines expressing Ile50 variant exhibit a phenotype with enhanced cell growth, Stat6 activation, and activation of Iε promoter upon IL-4 stimulation (50). The mechanism by which the Ile50 mutation in the extracellular domain of the IL-4 receptor augments signaling pathways mediated by the cytoplasmic tail without altering the ligand-binding affinity remains unknown. It is possible that Ile50 in the extracellular domain induces a conformational alteration in the cytoplasmic region of the IL-4Rα chain that favors prolonged activation of the signaling molecules. If this is to be valid, the conformational modification should have global effects on IRS and Stat6 signaling pathways.

Concluding remarks

IL-4 is the "prototypic immunoregulatory cytokine" and its biological responses and signaling pathways have been under intense investigation. The results of these investigations in the past decade have generated a clear picture, though not complete, of how various biological functions of IL-4 are mediated by different signaling pathways

through distinct domains of the IL-4Rα chain. In the past two years, novel regulatory elements, including the ITIM (and hence SH2-containing phosphatases), FRIP, and Suppressors of Cytokine Signaling (SOCSs) (52-55) have been added to the list of motifs/molecules involved in the IL-4 signaling pathway. These molecules introduce higher levels of complexity and potential mechanisms for fine-tuning to the IL-4 signaling pathway and warrant further studies. Moreover, two novel variants of the human IL-4Rα chains were identified from patients with atopy. These naturally occurring mutations of the IL-4Rα chain, one in the extracellular domain and the other in the gene activation domain, also open up a new arena for not only investigating the molecular genetic events that contribute to the development of allergies and asthma but also expanding our knowledge of the mechanisms underlying IL-4 signaling pathway. More thorough understanding of the signaling events mediated by IL-4 will provide the foundation for devising novel therapeutic strategies for allergies, parasitic infections, and autoimmune diseases.

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Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) of the IL-4 Receptor Associates with SH2-containing Phosphatases and Regulates IL-4 Induced Proliferation

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Running title: ITIM regulates IL-4 induced proliferation

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Summary

Immunoreceptor tyrosine-based inhibitory motifs (ITIM) have been implicated in the negative modulation of immunoreceptor signaling pathways. The IL-4 receptor α chain (IL-4Rα) contains a putative ITIM in the carboxyl terminal. Using peptides corresponding to the ITIM of human IL-4Rα (hIL-4Rα), we demonstrate that tyrosine-phosphorylated peptides, but not their nonphosphorylated counterparts, co-precipitate SH2-containing tyrosine phosphatase-1 (SHP-1) and SH2-containing inositol 5-phosphatase (SHIP). The *in vivo* association was verified by co-immunoprecipitation of the IL-4Rα and the two phosphatases with anti-IL-4Rα antibodies. Further analysis revealed that the tyrosine phosphorylation level of SHIP was increased upon IL-4 stimulation, whereas that of SHP-1 remained unchanged. To determine the role of the ITIM in the IL-4 signaling pathway, we ablated the ITIM of hIL-4Rα by deletion and site-directed mutagenesis, and stably expressed the wild-type (WT) and mutant hIL-4Rα in 32D cells. No significant difference in the duration of Stat6 activation was observed among cell lines expressing WT and mutant hIL-4Rα. However, 32D cells expressing mutant hIL-4Rα were hyperproliferative in response to IL-4 compared to cells expressing WT hIL-4Rα. The results indicate a functional role for the ITIM in the regulation of IL-4 induced proliferation.

Introduction

IL-4 is a pleiotropic cytokine that regulates growth and differentiation in various cell types of haemopoietic and non-haemopoietic origins (1). In hematopoietic cells, IL-4 exerts its multiple functions by binding to a high-affinity receptor complex composed of IL-4 receptor α chain (IL-4Rα) and common γ chain (γC) (2-5). Upon ligand engagement, the IL-4Rα is tyrosine-phosphorylated, creating docking sites for downstream signaling molecules. Five conserved tyrosine residues that can potentially be phosphorylated are present in the cytoplasmic domain of IL-4Rα (6). Phosphorylation of tyrosine 497 (Y1) has been shown to mediate mitogenic responses through recruitment and phosphorylation of insulin receptor substrate (IRS) 1 and/or 2 in non-haemopoietic and haemopoietic cells respectively (7-9). In contrast, IL-4 mediated gene activation and differentiation were mapped to a separate region of IL-4Rα. Tyrosine 575, 603 and 631 (Y2, Y3, and Y4) appear to be redundant binding sites for Stat6 (10, 11). Ablation of any one or two of the tyrosine residues does not significantly influence Stat6 phosphorylation and gene activation. However, deletion of all three tyrosine residues completely abolishes IL-4 mediated CD23 induction and Cε germline transcription in B cells.

The most C-terminal conserved tyrosine of IL-4Rα is Y716 (Y5) whose function in IL-4 mediated gene activation, growth and differentiation remains undetermined. The amino acid sequence flanking Y716 resembles the canonical consensus (I/VxYxxL) of the immunoreceptor tyrosine-based inhibitory motif (ITIM). The ITIM was first identified in the cytoplasmic domain of FcγRIIB and has been recognized in a growing family of

inhibitory receptors (12, 13). Its role in the negative modulation of immunoreceptor signaling has been suggested in FcγRIIB (14), killer cell inhibitory receptors (KIR) (15, 16), CD22 (17), and cytotoxic T-lymphocyte antigen-4 (CTLA-4) (18) signal pathways. Accumulating evidence reveals that the ITIM, when phosphorylated on tyrosine, becomes a docking site for SH2-containing tyrosine phosphatase-1 (SHP-1) and/or SH2-containing inositol 5-phosphatase (SHIP) (19-23). In addition, SH2-containing tyrosine phosphatase-2 (SHP-2) has been shown to be associated with the ITIM of FcγRIIB and CTLA-4 (19, 24-26). After recruitment to the inhibitory receptors, these phosphatases regulate signaling cascades initiating from B cell receptors, FcγRII, FcεRI, and T cell receptors, presumably by dephosphorylating activated signaling molecules. Furthermore, ITIMs have also been implicated in the regulation of cytokine signaling. The cytoplasmic domain of erythropoietin receptors (EPO-R) contains an ITIM, which mediates its regulatory role in proliferative signals by recruiting SHP-1 (27). Although the cytoplasmic domain of IL-4Rα contains a putative ITIM, its exact role in the regulation of IL-4 signaling pathway has not been studied. Here we report that SHP-1 and SHIP associate with the IL-4Rα ITIM and that the ablation of the ITIM results in a hyperproliferative response to IL-4 stimulation.

Materials and Methods

Antibodies , Cytokines and Peptides. Anti-SHIP antibody was generated as described previously (19, 28). Anti-SHP-1 was a gift from Dr. B. Neel (Harvard Medical School, Boston, MA). Anti-SHP-2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Shc antibody, anti-phosphotyrosine monoclonal antibody 4G10 and horseradish peroxidase-conjugated anti-rabbit Ig from Upstate Biotechnology (Lake Placid, NY), monoclonal anit-mIl-4Rα antibody and anti-hIL-4Rα antibody from R&D System (), and biotinylated goat anit-mouse Ig antibodies PE-conjugated anti-CD23 antibodies and avidin-conjugated APC from Pharmagen (). Murine IL-4 was a gift from Dr. R. Coffman (DNAX Research Institute, Palo Alto, CA) and human IL-4 from Dr. S. Narula (Schering-Plough Corp., Kenilworth, NJ). Biotinylated synthetic nonphosphorylated or tyrosine phosphorylated peptides corresponding to the sequence flanking the ITIM of human IL-4Rα were purchased from Quality Control Biochemicals, Inc. (Hopkinton, MA). The amino acid sequence of the peptide is SLGSGIVYSALTCHLC.

Expression Constructs. Human IL-4Rα deletion mutant (Δ715) was generated by restriction digestion of wild type (WT) hIL-4Rα (plasmids provided by Dr. William Paul, NIH, Bethesda, MD and Dr. J. Ryan, Virginia Commonwealth University, Richmond, VA) with *AccI* (New England Biolab, Beverly, MA). The Altered Sites® II *in vitro* Mutagenesis System (Promega, Madison, WI) was used to generate the point mutation (Y716F) of the critical tyrosine residue in the ITIM of hIL-4Rα per manufacturer's

instruction. The resulting fragments, Y716F, WT and Δ715 hIL-4Rα cDNA were blunt-ended and subcloned into MSCV-pIRES-GFP vector (provided by Dr. G. Nolan, Stanford University, Palo Alto, CA). The hIL-4Rα chain and GFP are expressed as a bicistronic message and internal ribosomal entry site (IRES) (29) allows concomitant expression of both hIL-4Rα and GFP.

Cell Culture and Retrovirus Production. M12 cells were cultured in complete RPMI-1640 (RPMI-1640 supplemented with 10% FCS (Sigma Chem. Co., St. Louis, MO), 2 mM L-glutamine, 50 μM 2-mercaptoethanol (Bio-Rad, Hercules, CA)). 32D cells stably expressing IRS-2 (32D/IRS-2; provided by Dr. J. Pierce, NIH, Bethesda, MD) were cultured complete RPMI-1640 supplemented with 5% WEHI-3 conditioned medium as a source of IL-3. ΦNX cell line for retrovius production (provided by Dr. G. Nolan) was grown in DMEM containing 10% FCS, 2 mM L-glutamine. All cell lines were maintained at 37°C in 5% CO_2. To produce retroviral stocks carrying hIL-4Rα genes, ΦNX cells were transfected with MSCV vectors by the calcium phosphate method. Supernatants containing virions were harvested 48 hours post-transfection. The transfection efficiency was measured by GFP expression of transfected ΦNX cells. Viral stocks were immediately used for infection or stored at -80°C.

Adsorption, Immunoprecipitation and Western Blot Analysis. 32D/IRS-2 cells were IL-3-starved for 4 hr and untreated or treated with murine IL-4 (100 ng/ml) for 30 min. M12 cells were stimulated with murine IL-4 for 10 min. Whole cell extracts were prepared with lysis buffer consisting of 0.5% NP-40, 50 mM Tris, pH 8.0, 10% glycerol,

0.1 mM EDTA, 150 mM NaCl, 100 μM Na_3VO_4, 50 mM NaF, 1mM DTT, 0.4 mM PMSF, 1 μg/ml leupeptin, 3 μg/ml aprotinin, and 2 μg/ml pepstatin. Biotinylated peptides corresponding to hIL-4Rα ITIM were incubated with Strept-avidin conjugated beads (Sigma Chem. Co.) at room temperature for 1 hr and subsequently free peptides were removed by washing 3 times with lysis buffer. Cell lysates were precipitated for 4 hr at 4°C with nonphosphorylated or phosphorylated peptides coupled to agarose beads. The adsorbed material was resolved on 10% SDS-PAGE and transferred onto nitrocellular membrane using semidry blotter (Bio-Rad). Immunoprecipitation and Western blot analysis were performed as described (30).

Generation of Stable Cell Lines Expressing Wild Type or Mutant hIL-4Ra. 32D/IRS-2 or M12 cells were infected with retroviral vectors carrying wild type (WT) or mutant (Δ715 and Y716F) hIL-4Rα genes according to the protocol described previously. Infected cell culture was expanded for 2-4 days before sorting. GFP positive cells were sorted by fluorescence activated cell sorter (FACStar, Becton Dickinson, Mountain View, CA). 32D/IRS-2 or M12 cells were also transduced with MSCV retroviral vector carrying only GFP cDNA and sorted for GFP positive cells to serve as a negative control cell line. Cell surface expression of hIL-4Rα was confirmed by immunofluorescent staining with mouse anti-hIL-4Rα monoclonal antibody (Genzyme, Cambridge, MA). The expression level of hIL-4Rα correlated with that of GFP.

Proliferation Assay. 32D/IRS-2 cell lines expressing hIL-4Rα chains were passaged 3 days before the experiment. On the day of the experiment, cells were washed 3

times with complete RPMI-1640 without 5% WEHI-3 conditioned medium, and then cultured at a density of 2.5 x 10^4 per 200 μl in a 96-well flat-bottom microtiter plate in culture medium with varying concentrations of recombinant hIL-4, or 5% WEHI-3 conditioned medium. Cells were pulse-labeled with 1μCi of [^{3}H]-Thymidine (specific activity 6.7 Ci/mmol; NEN, Boston, MA) for the last 6-8 hrs of the culture time and harvested at 7, 21, 30, and 53 hours after adding IL-4. [^{3}H]-Thymidine incorporation was measured by a scintillation counter.

Electrophoretic Mobility-Shift Assay. 32D/IRS-2 cells expressing WT or mutant hIL-4Rα chains were stimulated with 50 ng/ml human IL-4 (hIL-4) for 2, 24, 48, and 96 hours. Whole cell extracts were prepared and mobility-shift reactions were performed as described previously (31). The probe used was from IRF-1 GAS element: 5'-GATTCCCCGAAAT-3'.

Flow Cytometry Analysis. For surface expression of human IL-4 receptor, 32D/IRS-2 cells (1x106 cells) were stained with anit-human IL-4Rα monnoclonal antibodies for 30 min. on ice. After washing with PBS for 3 times, cells were incubated with biotinylated polyclonal anti-mouse Ig for 30 min. Cells were washed 3 times with PBS, then incubated with avidin conjugated APC (Pharmangen,) for 30 min, and washed with PBS. For CD23 induction by IL-4, M12 cells expressing WT or mutant hIL-4Rα (1x10^6 cells) were left unstimulated or stimulated with 10ng/ml human or murine IL-4 for 48 hrs, harvested, and stained with PE-conjugated anti-CD23 antibodies. Cells were analyzed by FACScan (Becton Dickinson, Mountain View, CA)

Results and Discussion

The cytoplasmic domain of IL-4Ra contains an ITIM. Tyrosine 716 of the IL-4Rα is the only conserved tyrosine whose function remains unknown. We were interested in the biological function of Y716 because the carboxyl terminal regions of human and murine IL-4Rα are highly conserved (Fig. 1A). Of particular interest is the region flanking Y716. When the amino acid sequence is aligned with the known ITIM sequences from CD22, KIR, FcγRIIB, and human EPO-R, a canonical consensus of the ITIM was apparent in the human and murine IL-4Rα chains (Fig. 1B). Among the five conserved tyrosine residues in the cytoplasmic region of IL-4Rα chain, only the sequence flanking Y716 resembles the consensus of the ITIM (I/VxYxxL).

SHP-1 and SHIP associate with the ITIM of hIL-4Rα. Although the IL-4Rα contains a putative ITIM, its role in the IL-4 signaling remains unexplored. It has been reported that SHP-1 could be co-precipitated with IL-4Rα (32). However, the nature of the association between IL-4Rα and SHP-1 is unclear. To determine whether the ITIM of IL-4Rα could associate with SH2-containing phosphatases such as SHP-1 and SHIP, the peptide corresponding to the ITIM of human IL-4Rα was synthesized. As shown in Fig. 2A, tyrosine phosphorylated peptides co-precipitated SHP-1 and SHIP in whole cell extracts prepared from unstimulated or IL-4 stimulated 32D/IRS-2 cells, whereas no interaction between the two phosphatases and nonphosphorylated peptides was observed. This is consistent with the tyrosine phosphorylation requirement for ITIM recruitment of SH2-containing phosphatases (15, 16). Two additional proteins on the anti-

phosphotyrosine blot were identified to be SHP-2 (upper band) and Shc (lower band) by blotting with anti-SHP-2 and anti-Shc antibodies (data not shown). To verify the *in vivo* association of IL-4Rα ITIM with SHIP, co-immunoprecipitation experiments with whole cell extracts were performed. Upon IL-4 treatment, SHIP was specifically associated with IL-4Rα chains (Fig. 2B). Moreover, the tyrosine phosphorylation of SHIP was enhanced with IL-4 stimulation (Fig. 2C), while that of SHP-1 remained unchanged (data not shown). A basal level of tyrosine phosphorylation in SHIP was observed in the whole cell lysate prepared from unstimulated cells.

Ablation of the IL-4Rα ITIM results in a hyperproliferative response to IL-4 stimulation. To assess the physiological role of the IL-4Rα ITIM, we generated 32D/IRS-2 cells expressing wild type (WT) hIL-4Rα and mutant IL-4Rα lacking the ITIM (Δ715 and Y716F). To avoid clonal variation among stable cell lines, we used GFP as a positive selection marker and FACS to collect cells expressing GFP and hIL-4Rα chains so that all stable cell lines used in this report are polyclonal in nature. We also took advantage of the fact that murine and human IL-4 do not cross-react so that endogenous murine IL-4Rα would not interfere with our analyses. Cell lines expressing WT or mutant IL-4Rα chains were sorted for comparable levels of GFP expression. The levels of surface receptor expression were confirmed by FACS analysis and correlated with that of GFP expression (Fig. 3A).

Previous reports on IL-3, c-kit, and EPO signaling pathways suggested an important role of ITIM in terminating proliferative signals (27, 33, 34). To determine

whether IL-4Rα ITIM played a regulatory role for proliferative signals induced by IL-4, we measured the proliferation of 32D/IRS-2 cells expressing WT and ITIM-deficient hIL-4Rα chains by [^{3}H]-thymidine incorporation assay over a time course of 7, 21, 30, and 53 hours. Upon stimulation with human IL-4 for 7 hours, no significant difference was observed among cell lines expressing WT or ITIM-deficient hIL-4Rα chains (data not shown). In contrast, at 21, 30 (data not shown), and 53 hours after IL-4 stimulation 32D/IRS-2 cells expressing ITIM-deficient IL-4Rα exhibited a hyperproliferative phenotype when compared to those expressing wild-type receptors. All cell lines reach maximal proliferation at 5 ng/ml of human IL-4. However, the maximal [^{3}H]-thymidine incorporation for cells expressing ITIM-deficient hIL-4Rα is 60,000 cpm at 21 hours after IL-4 stimulation whereas that for cells expressing WT hIL-4Rα is 40,000 cpm (Fig. 3B). As shown in Fig. 3C, at 53 hours after adding human IL-4 maximal [^{3}H]-thymidine incorporation for the ITIM-deficient IL-4Rα (Y716F and Δ715) is 2-fold higher than that of the WT IL-4Rα. The hyperproliferation was observed in concentrations ranging from 1 to 50 ng/ml human IL-4. The lower proliferative response to human IL-4 in 32D/IRS-2 cells expressing WT hIL-4Rα was not due to a defect in proliferative capability, for all three cell lines had similar levels of proliferation in the presence of 5% WEHI-3 conditioned medium at all time points examined (Fig. 3D). Hence, the ablation of ITIM in the cytoplasmic region of hIL-4Rα resulted in a hyperproliferative response to human IL-4, implicating a role for the IL-4Rα ITIM in the down-modulation of proliferative signals induced by IL-4.

We next determined whether the ITIM in the cytoplasmic tail of hIL-4Rα had a general regulatory effect on IL-4 signaling pathway by analyzing gene activation/differentiation signals in these cell lines. IL-4 receptors transduce gene activation/differentiation signals by recruiting and phosphorylating Stat6 (31). The phosphorylated Stat6 becomes homodimerized. The homodimerized Stat6 can bind to IFNγ activating sequence (GAS) element and be detected in an electrophoretic mobility shift assay. No significant difference in the duration of Stat6 activation was found between 32D/IRS-2 cells expressing WT hIL-4Rα and hIL-4Rα/Y716F upon IL-4 stimulation (Fig. 4). Stat6 GAS binding activities were detected up to 96 hr after IL-4 stimulation in both cell lines. Therefore, the IL-4Rα ITIM exerts no regulatory function on the duration of Stat6 activation. However, there appeared to be a slightly higher level of Stat6 GAS binding activities in extracts prepared from 32D/IRS-2 expressing hIL-4Rα/Y716F at 24, 48 and 96 hr time points. To further test whether the ITIM mutation had any effect on IL-4 mediated gene activation, we analyzed CD23 induction by IL-4 in M12 cells expressing WT and ITIM deficient hIL-4Rα. No significant difference in surface CD23 expression after IL-4 stimulation was observed between M12 expressing WT and ITIM-deficient hIL-4Rα (data not shown). This is consistent with previous findings that growth signal and gene activation/differentiation signal induced by IL-4 are independently regulated (10, 11).

While this manuscript was being prepared, a report by Zamorano and Keegan (35) implicated a regulatory role for the IL-4Rα C-terminal domain in IL-4-induced anti-apoptotic signaling. In agreement with our results, a slight increase in IL-4-induced

proliferation for hIL-4Rα/Y716 was observed at a single dose of 10 ng/ml human IL-4. In constrast, we observed a 2-fold increase in proliferation for ITIM-deficient hIL-4Rα (Δ715 and Y716F) at concentrations ranging from 5 to 50 ng/ml as compared to WT hIL-4Rα. However, we did not find any significant difference in IL-4 induced protection from apoptosis among cell lines expressing WT or ITIM-deficient human IL-4Rα chains (unpublished results, Pan, Giallouriakis, and Rothman).

The mechanism by which the IL-4Rα ITIM down-modulates IL-4 induced proliferation is yet to be studied. Since SHP-1 and SHIP associate with the IL-4Rα ITIM, it is possible that the IL-4Rα ITIM regulates IL-4 induced proliferation through the activities of these two SH2-containing phosphatases. SHP-1 has been shown to be recruited to the cytoplasmic tail of EPO-R and plays a major role in the down-modulation of proliferative signals induced by EPO (27). Upon EPO stimulation, SHP-1 specifically associates, via its SH2 domain, with phosphorylated Y429 in the EPO-R ITIM. The recruited SHP-1 then mediates its regulatory function by dephosphorylating and inactivating Jak2. How SHIP exerts its regulatory role remains to be elucidated. It has been proposed that SHIP contributes to anti-proliferative effect by competing with Grb2-Sos complex for binding to Shc, resulting in a block in the Ras signaling pathway (36-38). On the other hand, the enzymatic activity of SHIP may be required for its regulatory function. It has been shown that phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P_3) mediates the membrane association and activation of Tec and phospholipase C (39). By removing 5-phosphate from PI-3,4,5-P_3, SHIP blocks the accumulation of PI-3,4,5-P_3,

thereby inhibiting the sustained calcium influx required for calcium-dependent proliferative responses (40).

To further test whether SHIP plays a regulatory role in IL-4 signaling, we have established 32D/IRS2 cells overexpressing wild type and phosphatase-deficient SHIP. Interestingly, cells overexpressing wild type SHIP have a hyperproliferative phenotype whereas those overexpressing phosphatase-deficient SHIP display the exactly opposite phenotype (Giallourakis et al., submitted). These results suggest a positive regulatory role for SHIP in IL-4 induced proliferation, seemingly contradicting the results presented here that SHIP associates with the IL-4Rα ITIM and that the IL-4Rα ITIM down-modulates IL-4 induced proliferation. It has been shown that SH2-containing phosphotases can be recruited to other motifs of IL-4Rα in addition to the IL-4Rα ITIM (Ref). It is possible that by binding to distinct motifs of IL-4Rα and interacting with different components of IL-4 signaling complex, SHIP may exert different regulatory functions on IL-4 signaling. Alternatively, the hyperproliferative phenotype observed in 32D/IRS-2 expressing ITIM-deficient hIL-4Rα might not result from being unable to recruit SHIP but from the absence of an unidentified ITIM-binding protein. While it is not yet clear how the IL-4Rα ITIM regulates IL-4 induced proliferation in 32D/IRS-2 cells, the data presented here strongly suggest a functional ITIM in the C-terminal of human IL-4Rα chains.

In summary, we have identified a functional ITIM in the cytoplasmic region of IL-4Rα chain. The IL-4Rα ITIM associates with two SH-2 containing phosphatases and

regulates proliferative signals induced by IL-4. Our results demonstrate a new regulatory element in the IL-4 signaling pathway.

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Figure 1. Amino acid sequences of human and murine IL-4Rα chain carboxyl terminals and ITIM sequences. (A) Sequence alignment of carboxyl terminals of human and murine IL-4Rα chains. The amino acid sequence (708-770) of human IL-4Rα is compared with the corresponding region of the mouse IL-4Rα. The putative ITIM sequence of IL-4Rα is underlined. The amino acid residues of murine IL-4Rα identical to the human IL-4Rα are marked "-". (B) Alignment of ITIM sequences of CD22, KIR, FcγRIIB, EPO-R, and IL-4Rα. The ITIM consensus is boldfaced. The single amino acid code is used and X represents any amino acid.

Figure 2. The association of SHP-1 and SHIP with the tyrosine phosphorylated IL-4Rα ITIM and the induction of tyrosine phosphorylation of SHIP by IL-4 stimulation. (A) SHP-1 and SHIP bind the phosphorylated ITIM of IL-4Rα chain. Biotinylated peptides were incubated with Strep-avidin-conjugated agarose beads. Cleared whole cell lysates prepared from $1x10^8$ M12 cells were incubated with nonphosphorylated (Y) and phosphorylated (pY) peptides coupled to agarose beads. Material adsorbed by peptides was resolved by 7% SDS-PAGE, transferred onto nitrocellular membrane, and subsequently immunoblotted with anti-phosphotyrosine, anti-SHIP, and anti-SHP-1 antibodies. (B) SHIP is co-immunoprecipitated with IL-4Rα chain by anti-IL-4Rα antibodies. Immunoprecipitations of lysates prepared from unstimulated and stimulated M12 were performed using anti-mIL-4Rα, anti-SHIP and normal rabbit serum (NRS). Immunoprecipitates were resolved by 7% SDS-PAGE, and immunoblotted with anti-SHIP

antibody. The same blot was striped and reprobed with anti-mIL-4Rα antibody. (C) Tyrosine phosphorylation of SHIP is enhanced by IL-4 stimulation. M12 cells were left untreated or treated with 100 ng/ml murine IL-4 for 30 min and whole cell extracts were prepared. Immunoprecipitations were performed using anti-SHIP antibody. Immunoprecipitates were separated by 7% SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody. Membrane was subsequently stripped and reprobed with anti-SHIP antibody to confirm equal loading of protein.

Figure 3. IL-4 induced proliferation of 32D/IRS-2 cells expressing wild type and mutant human IL-4Rα chains. (A) Surface human IL-4Rα expression by 32D/IRS-2 cells expressing WT or ITIM-deficient hIL-4Rα. Cells were stained with anti-human IL-4Rα mouse monoclonal antibodies, followed by incubating with biotinylated goat anti-mouse Ig and Avidin-conjugated APC. GFP positive cells were gated and analyzed for surface staining of human IL-4Rα chains. (B) Proliferative responses of 32D/IRS-2 cells expressing WT, Δ715, or Y716F human IL-4Rα stimulated with various concentrations of human IL-4 for 21 hrs. (C) Proliferative responses of 32D/IRS-2 cells expressing WT, Δ715, or Y716F human IL-4Rα stimulated with various concentrations of human IL-4 for 53 hrs. (D) [^{3}H]-thymidine incorporation of 32D/IRS-2 cells expressing various hIL-4Rα constructs in the presence of 5% WEHI-3 conditioned medium as a source of IL-3. The figures show the proliferation of 1 representative out of 4 independent experiments. Each data point represents the mean ± standard deviation of triplicate counts.

Figure 4. Induction of Stat6 GAS-binding activity in 32D/IRS-2 cells expressing wild type or Y716F human IL-4Rα chains. Cells were washed 3 times with complete RPMI-1640 to remove IL-3 and were left untreated or treated with 50 ng/ml human IL-4 for the time indicated. Whole cell extracts were prepared and analyzed by EMSA using IRF-1 GAS element as a probe. Stat6 GAS-binding activity was detected throughout the time points analyzed in cells expressing wild type or Y716F human IL-4Rα chains.

A

716

Human 708 D S L G S G I V Y S A L T C H L C G H L K Q C H G Q E D G G Q
Mouse - D - - F - - - - - S - - - - - - - - - - - H - S - - E - - -

Human 739 T P V M A S P C C G C C C G D R S S P P T T P L R A P D P S P G
Mouse S - I V - - - G - - - - Y D - - - P S L G S L S G A L E S C - E

B

hCD22 (Y2)	E D G **I** S **Y** T T **L** F F
hCD22 (Y5)	D E G **I** H **Y** S E **L** I Q
hCD22 (Y6)	Q E N **V** D **Y** V I **L** K H
hKIR (N-terminal)	P Q E **V** T **Y** A Q **L** N H
hKIR (C-terminal)	T D I **I** V **Y** A E **L** P N
hFcγRIIB	E N T **I** T **Y** S L **L** M H
mFcγRIIB	E N T **I** T **Y** S L **L** K H
hEPO-R	P P H **L** K **Y** L Y **L** M H
hIL-4Rα	G F G **I** V **Y** S S **L** T C
mIL-4Rα	G S G **I** V **Y** S A **L** T C
ITIM consensus	**I/V X Y X X L**

Fig. 1.